Campylobacter spp. and *Arcobacter spp.* in Locally Produced Raw Chicken: Isolation, Identification, Characterization and Control Strategies

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This dissertation has been submitted to the Council of Agrarian Sciences of the Agricultural University of Georgia to seek the academic degree of Doctor of Agrarian Sciences

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საქართველოს აგრარული უნივერსიტეტი
სამეცნიერო მიმართულების კომისიის რეკომენდაცია

დისერტანტი: ლეონიდ უშანოვი

დისერტაციის სათაური: "*Campylobacter spp.* და *Arcobacter spp.* ადგილობრივი წარმოების ქათმის ნედლ ხორცში: გამოყოფა, იდენტიფიკაცია, დახასიათება და კონტროლის სტრატეგიები".

"*Campylobacter spp.* and *Arcobacter spp.* in Locally Produced Raw Chicken: Isolation, Identification, Characterization and Control Strategies".

დისერტაციის დაცვის თარიღი: 12 თებერვალი, 2024

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სადისერტაციო კომისიის თავმჯდომარე: ვლადიმერ ელისაშვილი

სადისერტაციო კომისიის წევრი: ლალი ქუთათელაძე

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სადისერტაციო კომისიის წევრი: ანდრეას ერიხ ზაუტნერ

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Table of Contents

Table of Figures

Author's declaration

As the author of the submitted doctoral dissertation, I declare that this dissertation is an original work. All materials previously published, or defended by other authors are used in this dissertation in accordance with the proper citation rules.

Leonid Ushanov

Acknowledgments

I would like to thank my PhD advisors and my colleagues for their backing and support:

Professor Dr. Dr. h.c. mµLt. Angelika Ploeger, Sustainable Agriculture and Food Systems (SAFS), Project Director – VW, Kassel University, Germany

Professor Dr. Andreas Erich Zautner – OvG University of Magdeburg, Germany

Professor Vakhtang Lezhava, Rector of the Agricultural University of Georgia

Professor Dr. Teo Urushadze, Dean of Agricultural and Nature Science School at the Agricultural University of Georgia, Director of SAFS Georgia Program

Natia Samushia Vice-Rector of the Agricultural University of Georgia

Professor Nato Kobakhidze, PhD. School coordinator of the Agricultural University of Georgia

Nino Katcharava, SAFS program coordinator

List of Abbreviations

CaCo-2	Carcinoma Colon-2 cell line (Human)
C-broth	Composite broth devised for the purpose of this research
CCDA	Cefoperazone Charcoal Deoxicholate Agar
COG	Clusters of Orthologous Groups
DMEM	Dulbecco's modified Eagle Medium
EFSA	European Food Safety Agency
EMA	European Medicines Agency
EU	European Union
GBS	Guillain Barré Syndrome
HGT	Horizontal Gene Transfer
LAB	Lactic Acid Bacteria
MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time of Flight
MH	Mueller Hinton
MDR	Multidrug Resistance
MRS	Mann Rogosa Sharpe
NGS	Next Generation Sequencing
NCTC	National Collection of Tissue Culture
OD	Optical Density
SDG	Sustainable Development Goal(s)
SAFS	Sustainable Agriculture and Food Systems
UN	United Nations
US	United States
US FDA	The United States Food and Drug Administration

ლეონიდ უშანოვის

სადისერტაციო ნაშრომის

Campylobacter spp. და *Arcobacter spp.* ადგილობრივი წარმოების ქათმის ნედლ ხორცში: გამოყოფა, იდენტიფიკაცია, დახასიათება და კონტროლის სტრატეგიები

ანოტაცია

წარმოდგენილი სამუშაო მიზნად ისახავდა ზოონოზური პათოგენების-*Campylobacter* spp. და *Arcobacter* spp. დეტექციას ქათმის ნედლ სარეალიზაციო ხორცში. კერძოდ, კვლევის ფარგლებში განხორციელებულ იქნა შემდეგი სამუშაოები:

1. დადგენილ იქნა სარეალიზაციო ქათმის ხორციდან გამოყოფილი *Campylobacter* spp. და *Arcobacter* spp. და მათი ანტიბიოტიკების მიმართ მგრძნობელობა.
2. ფილოგენეტიკური ანალიზის შედეგად ნაჩვენები იქნა, რომ კვლევის ფარგლებში გამოყოფილი და შესწავლილი *Arcobacter*-ის შტამები მიეკუთვნებიან ახალ სახეობას, რომელისთვისაც მინიჭებულ იქნა სახელი *Arcobacter tbilisiensis* sp. nov. ტიპური შტამი LEO 51, რეგისტრირებულია გერმანულ პროკარიოტული და ცხოველური უჯრედების ბანკში DSMZ (German Collection of Microorganisms and Cell Cultures, Leibniz Institute) როგორც DSM 11590 და ბელგიურ უჯრედების კოლექციაში BCCM (Belgian Coordinated Collection of Microorganisms) როგორც LMG 33177.
3. კოინკუბაციისა და ციტოტოქსიკური ექსპერიმენტების საშუალებით ნაჩვენები იქნა, რომ *Campylobacter*-ის ორივე დახასიათებული სახეობა-*C. jejuni* და *C. coli*-ისევე როგორც *Arcobacter*-ის დახასიათებული ერთი სახეობა-*A. Tbilisiensis*- ითრგუნება პრობიოტიკ *L. fermnentum*-თან ერთ საკვებ არეში ინკუბირების შემდგომ. ზემოთაღნიშნულმა დაკვირვებამ აჩვენა, რომ *L. fermentum* წარმოადგენს *Campylobacter*-ის და *Arcobacter*-ის ეფექტურ დამთრგუნველს in vitro პირობებში.

კვლევის აქტუალობა

წარმოდგენილი კვლევა საქართველოში *Campylobacter* spp. და *Arcobacter* spp. სარეალიზაციო ქათმის ხორციდან კულტივირების და მათი დახასიათების პირველი მაგალითია. ცნობილია, რომ *Campylobacter* spp. ძირითად რეზერვუარს სწორედ ქათმის ხორცი და ფერმის პირობებში გაზრდილი ქათამი წარმოადგენს. აღსანიშნავია, რომ აქამდე არ ყოფილა შესწავლილი საქართველოში გავრცელებულ დიარეულ დაავადებებს შორის რა ხვედრითი წილი აქვს კამპილობაქტერიოზს და *Arcobacter* spp.-ით გამოწვეულ ინფექციებს. თუმცა, ჩვენი კვლევის შედეგებზე დაყრდნობით, შეგვიძლია ვივარაუდოთ, რომ აღნიშნული მაჩვენებელი საკმაოდ მაღალი უნდა იყოს. აღსანიშნავია, რომ *Campylobacter* spp. კონტროლის აუცილებლობიდან გამომდინარე, მნიშვნელოვანია ასეთი კონტროლი განხორციელდეს კომბინირებული მეთოდებით, როგორიცაა მკაცრი სანიტარული ნორმების, ბიოუსაფრთხოებისა და ბიოლოგიურად აქტიური პრობიოტიკული საკვები დანამატების ერთობლიობა.

ყოველივე ზემოთქმულიდან გამომდინარე, ბიოლოგიურად აქტიური საკვები დანამატის შექმნა და აგრარულ სფეროში უვნებელი ქათმის ხორცის წარმოება წარმოადგენს იმ აქტუალურ საკითხს, რომელიც საქართველოში ჯერ-ჯერობით გადაწყვეტილი არ არის.

კვლევის სიახლე მდგომარეობს *Campylobacter* spp. და *Arcobacter* spp. კულტივირებასა და დახასიათებაში და ამასთანავე ამ პათოგენებზე პრობიოტიკ *L. fermentum*-ი დამთრგუნველი მოქმედების შესწავლაში. კვლევის მნიშვნელოვანს სიახლეს წარმოადგენს *A. tbilisiensis* sp. nov. ყველა შტამის გენომების სეკვენირება, რაც განხორციელდა პირველად.

ჩატარებული კვლევა მიზნად ისახავდა ქართულ ნედლ სარეალიზაციო ქათმის ხორცში *Campylobacter* spp. და *Arcobacter* spp. გავრცელების შეფასებას და ასევე, კულტივირებული შტამების ვირულენტობისა და ანტიბიოტიკების მიმართ რეზისტენტობის დახასიათებას. კვლევის შედეგად გამოვლინდა, რომ, ისევე როგორც მსოფლიოს სხვა ქვეყნებში, ქართული ნედლი ქათმიდან კულტივირებული *Campylobacter*-ის სახეობები, როგორიცაა *C. coli* და *C. jejuni*, ხასიათდებიან ციპროფლოქსაცინისადმი მაღალი რეზისტენტობით (97% და 79%). ამასთანავე, კვლევა მიზნად ისახავდა ქათმის საკვებში ბიოლოგიურად აქტიური დანამატების გამოყენებით ქათამში *Campylobacter* spp. შემცველობის კონტროლს. In vitro ექსპერიმენტებში, *L. fermnentum*-ის სპეციფიკური დამთრგუნველი აქტივობიდან გამომდინარე, მოსალოდნელია მსგავსი აქტივობის გამოვლენა in vivo პირობებშიც. აღნიშნული ვარაუდის დადასტურება მოითხოვს დამატებით კვლევებს მომავალში.

მომხმარებლის უვნებელი საკვებით მომარაგება პირდაპირ შეესაბამება მდგრადი განვითარების მე-3 მიზანს-„ჯანმრთელობა და კეთილდღეობა“, რომელიც აერთიანებს რამდენიმე მნიშვნელოვან ინდიკატორს, მათ შორის ინდიკატორს 3.3, რომელიც მიზნად ისახავს 2030 წლამდე აღმოფხვრას ან მნიშვნელოვანად შეამციროს ტუბერკულოზი, ჰეპატიტი, მალარია და სხვა გადამდები და ტროპიკული დაავადებები. ასევე, უვნებლობის საკითხი მჭიდრო კავშირშია მე-2 (შიმშილობის აღმოფხვრა) და მე-9 (ინდუსტრია, ინოვაცია და ინფრასტრუქტურები) მიზანთან. მდგრადი განვითარების მიზნების პრიორიტეტულობის შესახებ, რაც მნიშვნელოვანია 2030 წლისათვის მდგრადი მომავლის მიღწევის საქმეში, თანხმდება გაეროს ყველა წევრი ქვეყანა.

სამეცნიერო ლიტერატურის მიმოხილვა

სამეცნიერო ლიტერატურის მიმოხილვა მოიცავს სამეცნიერო პუბლიკაციებს მსოფლიოს სხვადასხვა ქვეყანაში *Campylobacter* spp. და *Arcobacter* spp. ეპიდემიოლოგიის შესახებ. მსოფლიოს ბევრ ქვეყანაში, მათ შორის ევროკავშირსა და აშშ-ში, კამპილობაქტერიოზი წარმოადგენს დიარეული სინდრომების წამყვან

დაავადებას, რაც სარეალიზაციო ქათმის ხორცში *Campylobacter* spp. ეპიდემიოლოგიას უკავშირდება. ბოლო ათწლეულში, ასევე, ნაჩვენები იქნა, რომ *Arcobacter* spp. ხვედრითი წილი საკმაოდ მომატებულია დიარეულ სინდრომებში.

ხსენებული ზოონოზი, *Campylobacter* spp. მსგავსად, იწვევს დიარეულ სინდრომს. *Arcobacter* spp. ინფიცირების შემთხვევები მსოფლიოში იმდენად გახშირდა, რომ გაეროომ მას ეპიდემიოლოგიური თვალსაზრისით მაღალი რისკის პათოგენის კვალიფიკაცია მიანიჭა.

ბოლო ათწლეულში *Campylobacter* spp. გამოწვეულმა დიარეული დაავადებების სიხშირემ გაუსწრო *Salmonella* spp. და *E. coli*-ს შემთხვევების სიხშირეს ევროკავშირსა და აშშ-ში. დღეისათვის შეინიშნება ადამიანებში კამპილობაქტერიოზის სამკურნალოდ გამოყენებადი ანტიბიოტიკ ერითრომიცინის მიმართ *C. coli*-სა და *C. jejuni*-ს რეზისტენტობის მზარდი ტენდენცია. ორი ათეული წლის წინ კამპილობაქტერიოზის სამკურნალოდ წარმატებით და ინტენსიურად გამოიყენებოდა ციპროფლოქსაცინი, თუმცა ბაქტერიის მიერ გამომუშავებული რეზისტენტობის გამო ეს ანტიბიოტიკი ამჟამად არ არის რეკომენდირებული. ზემოთთქმულიდან გამომდინარე, თანამედროვე მსოფლიოს როგორც განვითარებული, ასევე განვითარებადი ქვეყნების წინაშე მწვავედ დგას სარეალიზაციო ქათმის ხორცში *Campylobacter* spp. რაოდენობის კომპლექსური ღონისძიებების საშუალებით შემცირების საკითხი. წარმოდგენილი ნაშრომის ლიტერატურის მიმოხილვის ნაწილში ყურადღება გამახვილებულია სწორედ ასეთ კომპლექსურ ღონისძიებებზე. ამ უკანასკნელის მაგალითს წარმოადგენს *Campylobacter* spp. გამოვლენა ფერმის პირობებში გაზრდილ წიწილებში და ყველა ინდივიდის დაინფიცირების თავიდან ასარიდებლად მათი დაყოფა კამპი-დადებით და კამპი-უარყოფით ჯგუფებად. ამ ღონისძიებათაგან უმნიშვნელოვანესია მკაცრი სანიტარული ნორმები, სუფთა წყალი (დაბინძურებული წყალი წარმოადგენს *Campylobacter* spp. გავრცელების ერთ-ერთ მნიშვნელოვან ფაქტორს) და აქტიური საკვები დანამატები. სადისერტაციო კვლევის ფარგლებში რძემჟავაბაქტერია *L. fermnentum*-მა გამოავლინა *Campylobacter*-ის მაღალი ეფექტურობით ინჰიბირება, რამაც ამ პრობიოტიკის აქტიურ საკვებ დანამატად გამოყენების პოტენციალი აჩვენა. ასევე, *L. fermnentum*-მა მოახდინა

ადამიანის ნაწლავის ეპითელიუმის CaCo-2 უჯრედების პროტექცია *Campylobacter* spp.-ით ინფიცირებისგან in vitro პირობებში. ეს მიანიშნებს იმაზე, რომ ინჰიბირება დიდი ალბათობით წარმატებით განმეორდება in vivo პირობებშიც.

ლიტერატურის მიმოხილვაში, ასევე, განხილულია მეცნიერთა სხვადასხვა ჯგუფების მიერ *Lactobacillus* spp. და სხვა პრობიოტიკების გამოყენებით ჩატარებული კვლევები. ჩვენ ექსპერიმენტებში გამოვრიცხეთ pH ფაქტორი სპეციალური კომბინირებული საკვები არის გამოყენებით. შესაბამისად, *C. jejuni და C. coli*-ის დათრგუნვა მოხდა ბაქტერიოცინის გამომუშავების მექანიზმით, რაც უფრო სპეციფიკური და ეფექტური მექანიზმია მსგავსი პროცესების განსახორციელებლად in vivo. აღნიშნული მიდგომა მნიშვნელოვანია იმდენად, რამდენადაც ლიტერატურული მონაცემების თანახმად, in vitro ექსპერიმენტებში რძემჟავა ბაქტერიების უმრავლესობას აქვს უნარი დათრგუნოს *Campylobacter* spp. და სხვა გრამ-უარყოფითი პათოგენები, რაც გამომდინარეობს ამ ბაქტერიების მიერ ორგანული მჟავების-რძემჟავისა და ძმარმჟავის-პროდუცირებიდან. საკვებ არეში გამოყოფილი ორგანული მჟავები, აკუმულაციის გამო და დაბალი pH-ის წარმოქმნის შედეგად, თრგუნავენ სხვადასხვა პათოგენებს. ცნობილია, რომ in vivo პირობებში pH რეგულირდება ორგანიზმის ჰომეოსტაზით. ქათმის ნაწლავის pH შეადგენს 5.5-ს, რაც არ წარმოადგენს რაიმე პრობლემას *Campylobacter* spp.-ისთვის. ჯერ-ჯერობით დადგენილი არ არის, რამდენად ეფექტური იქნება *Campylobacter* spp.-ზე მხოლოდ pH-დამოკიდებული მექანიზმის მეშვეობით მოქმედი რძემჟავა ბაქტერიების გამოყენება. აღსანიშნავია, რომ გარდა ანტიპათოგენური ზემოქმედებისა, *Lactobacillus* spp. ასევე ავლენენ სხვა სასარგებლო თვისებებს, როგორიცაა, მაგალითად, ცხოველის იმუნური სისტემის მოდულაცია.

კვლევის მეთოდები

კვლევის ძირითადი ობიექტი: ქართული სარეალიზაციო ქათმის ხორციდან კულტივირებული *Campylobacter* spp. და *Arcobacter* spp. სხვადასხვა შტამი. კვლევისათვის აუცილებელი რძემჟავაბაქტერიის 37 სხვადასხვა სახეობის შტამი

მოწოდებულ იქნა გ. ელიავას სახელობის ბაქტერიოფაგის, მიკრობიოლოგიისა და ვირუსოლოგიის ინსტიტუტის მიერ. *L. plantarum*-ის 10 შტამი მოგვაწოდა საქ. აგრარული უნივერსიტეტის ს. დურმიშიძის სახ. ბიოქიმიისა და ბიოტექნოლოგიის ინსტიტუტის პროკარიოტებისა და საფუვრების ლაბორატორიამ. ჩვენს მიერ სხვადასხვა წყაროდან (მაგ. მწნილებიდან-*L. plantarum*-ი, ხაჭოდან *L. casei* და ა. შ.) გამოყოფილ იქნა რძემჟავაბაქტერიის 5 სხვადასხვა სახეობა, მათ შორის *L. fermentum*-ი. ექსპერიმენტებისათვის შევარჩიეთ *C. jejuni*-ს, *C. coli*-ს და *A. tbilisiensis*-ის შტამები.
ქათმის ხორცის ნიმუშებიდან *Campylobacter* spp.-ისა და *Arcobacter* spp.-ის კულტივირება განხორციელდა სტანდარტული მეთოდით CCDA აგარის გამოყენებითა და მასში ანტიბიოტიკების დამატებით. ხშირ შემთხვევაში საჭირო იყო ნიმუშების პრე-ინკუბაცია ბოლტონის საკვებ არეში.

რძემჟავაბაქტერიების კულტივირება განხორციელდა MRS აგარის გამოყენებით.

რძემჟავაბაქტერიებისა და *Campylobacter* spp.-ის კოინკუბაცია შესაძლებელი გახდა ჩვენს მიერ შემუშავებული ინოვაციური მიდგომით, რომელიც გულისხმობდა კომბინირებულად M17 და MH საკვები არეების გამოყენებას (25%/75%) საკვებ არეში ნეიტრალური pH-ის შესანარჩუნებლად.

Campylobacter-ის და *Arcobacter*-ის სახეობების დასადგენად გამოყენებულ იქნა MALDI-TOF მასური სპექტრომეტრია.

ანტიბიოტიკოგრამა გაკეთდა შემდეგი ანტიბიოტიკების გამოყენებით (Oxoid): kanamycin (30), penicillin G (10), ciprofloxacine (5), erythromycin (15), gentamicin (30), chloramphenicol (30), ampicillin (10), streptomycin (25).

Campylobacter-ის და *Arcobacter*-ის სხვადასხვა შტამის გენომების სექვენირება ჩატარებულ იქნა Illumina MiSeq-ის და Oxford Nanopore პლატფორმებით.

ციტოტოქსიკურობის განსაზღვრისათვის გამოყენებულ იქნა Roche/Sigma Aldrich WST1 რეაქტივი, რომელიც ციტოტოქსიკურობის ეფექტს ავლენს უჯრედებში ტეტრაზოლიუმ მარილის გახლეჩვით, რის შედეგადაც წარმოიქმნება მოწითალო-მოყავისფრო შეფერილობის ნივთიერება. ტეტრაზოლიუმის გახლეჩვის უნარი აქვთ მხოლოდ მეტაბოლურად აქტიურ უჯრედებს, შესაბამისად ასეთი აქტიურობა არ ექნებათ დაზიანებულ უჯრედებს. ინკუბაციის შემდგომ, მიკროპლანშეტზე, სადაც მოთავსებულია უჯრედები *Campylobacter*/*Arcobacter*-თან ერთად და მის გარეშე, დაიტანება WST1 რეაქტივი 10 მკლ-ის ოდენობით. 30 წუთიდან–2 საათამდე ინუბაციის შემდგომ რეაქცია იზომება სპექტროფოტომეტრის საშუალებით და ხდება ციტოტოკსიკურობის ოდენობის კალკულაცია ნეგატიური კონტროლის და ფონის (background) შთანთქმური ღირებულებების გათვალისწინებით.

ექსპერიმენტული ნაწილი

ექსპერიმენტული ნაწილი მოიცავდა 4 ეტაპს. კერძოდ:

1. პირველი ეტაპი *Campylobacter* spp. კულტივაცია ნედლი ქათმის ხორციდან. ამ ეტაპზე მნიშვნელოვანი იყო ნიმუშის პრე-ინკუბაცია, შესაბამისი ფორმისა და შეფერილობის მქონე კოლონიების დეტექცია და შეღებვა *Campylobacter* spp.-თვის დამახასიათებელი მორფოლოგიის დასადგენად.
2. მეორე ეტაპი-კულტივირებული *Campylobacter* spp. შტამების კოინკუბაცია რძემჟავაბაქტერიებთან და რძემჟავაბაცტერიების იმ შტამების განსაზღვრა, რომლებიც თრგუნავენ *Campylobacter* spp. in vitro პირობებში. ამ ეტაპზე, ასევე, ვახდენდით რძემჟავაბაქტერიების ახალი შტამების გამოყოფას.
3. მესამე ეტაპი–გამოყოფილი *Campylobacter-ის* და *Arcobacter*-ის შტამების სახეობების დადგენა მასური სპექტრომეტრიის საშუალებით, მათი ანტიბიოტიკების მიმართ მგრძნობელობის დადგენა.

4. მეოთხე ეტაპი ყველა *A. tbilisiensis* შტამის (n=19) სექვენირება და მონაცემების დამუშავება. შედეგების სტატისტიკური დამუშავებისათვის გამოყენებულ იქნა პროგრამა JASP (Version 0.16.3).

შედეგები

1. ნაჩვენები იქნა, რომ ქათმის ხორციდან კულტივირებული 93 შტამი შეიცავს *Campylobacter*-ის ორ და *Arcobacter*-ის ერთი სახეობას. კერძოდ:

- *C. jejuni*, 39 შტამი
- *C. coli*, 35 შტამი
- *A. tbilisiensis*, 19 შტამი

2. ანტიბიოტიკების მგრძნობელობის მეთოდით ნაჩვენები იქნა, რომ *Campylobacter* spp. ყველა შტამი არის პენიცილინისადმი რეზისტენტული, ხოლო ციპროფლოქსაცინისადმი რეზისტენტულია *C. coli* -ს 97% და *C. jejuni*-ს 79%, რაც ორივე შემთხვევაში მაღალი რეზისტენტობის მაჩვენებელია. ასევე, მაღალი რეზისეტენტობა გამოავლინდა ტეტრაციკლინისადმი. ამ ანტიბიოტიკისადმი რეზისტენტულია *C. coli* -ს 51%, ხოლო *C. Jejuni*-ს 28%.

3. ნაჩვენები იქნა, რომ *A. tbilisiensis* 22% რეზისტენტულია ტეტრაციკლინის მიმართ, 44% ავლენს რეზისტენტულობას ამპიცილინის მიმართ, ხოლო პენიცილინის მიმართ რეზისტენტულია *A. tbilisiensis*-ის ყველა შტამი.

4. *Campylobacter*-ისა და *Lactobacilli*-ს კო-ინკუბაცია M17/MH საკვებ არეში (C-broth) მიმდინარეობს საკვები არის ნეიტრალური pH-ის შენარჩუნებით.

5. გამოვლინდა, რომ *L. fermentum* აინჰიბირებს *Campylobacter*-ის ორივე სახეობას (*C. jejuni* და *C. Coli)* in vitro 24 საათის განმავლობაში კოინკუბაციის შემდეგ. უარყოფითი კონტროლისა და სხვა ლაქტობაცილებთან კოინკუბაციის ნიმუშებისაგან განსხვავებით, შეუძლებელი ხდება *Campylobacter* spp. ამოთესვა სპეციფიურ აგარზე (CCDA).

6. ციტოტოკსიკურობის WST1 მეთოდის გამოყენებით ნაჩვენები იქნა, რომ როგორც *Campylobacter*-ის, ასევე *Arcobacter*-ის სხვადასხვა შტამი (115, 99, 38,104,105 და 106)

უარყოფითად მოქმედებს ადამიანის ნაწლავის ეპითელურ უჯრედებზე (CaCo-2) in vitro პირობებში. ციტოტოქსიკურობა მერყეობს 52-83% მდე.

7. ციტოტოკსიკურობის WST1 მეთოდით, ასევე, ნაჩვენებია, რომ *L. fermentum*-ის თანდასწრებით, ზემოთხსენებული შტამები ვეღარ ახერხებენ CaCo-2 უჯრედების დაზიანებას. მაშასადამე, ციტოტოკსიკურობა ან საერთოდ არ აღინიშნება, ან მისი მაჩვენებელი უმნიშვნელოა.

მიღებული შედეგები ცხადყოფს, რომ, *Campylobacter*-ის ორი სახეობა-*C. coli* და *C. Jejuni*, მაღალი სიხშირით არის გავრცელებული ადგილობრივი წარმოების ქათმის ნედლ ხორცში. კოინკუბაციის და შემდგომი in vivo კვლევების შედეგებზე დაყრდნობით, დიდი ალბათობით შესაძლებელი გახდება ლაქტობაცილებისგან შემდგარი უსაფრთხო კოქტეილის შემუშავება. ხსენებული კოქტეილის საშუალებით კი სავარაუდოდ ბროილერების ნაწლავებში და, შესაბამისად, ტან-ხორცზე *Campylobacter* spp.-ის ხვედრითი წილი შემცირება.

დასკვნები და რეკომენდაციები

წარმოდგენილი ნაშრომი მიმოიხილავს კომპლექსური ზომების საშუალებით *Campylobacter* spp. კონტროლის შესაძლებლობას. აღნიშნულ კომლექსურ ზომებში შედის როგორც ფერმის პირობების გაუმჯობესება ფრინველებისათვის, ასევე ბიოკონტროლი, პერიოდული *Campylobacter* spp. ტესტირება და კონტროლი, ფრინველებისათვის სუფთა წყლის მიწოდება და ასევე პრობიოტიკული საკვები დანამატი, რომელიც ხელს შეუწყობს *Campylobacter* spp. -ის რაოდენობის შემცირებას.

ჩვენს მიერ ჩატარებული ექსპერიმენტული კვლევების საფუძველზე შემუშავებულია *Campylobacter*-ის დათრგუნვის სტრატეგია in vitro. სამომავლო კვლევების მიზანს შეადგენს აღნიშნული სტრატეგიის გამოცდა in vivo სისტემაში.

სადისერტაციო კვლევის ფარგლებში მიღებული შედეგები მნიშვნელოვანი და საინტერესოა არა მხოლოდ სამეცნიერო, არამედ კომერციული თვალსაზრისითაც. კერძოდ, დაგეგმილია *L. fermentum*-ის მიერ *Campylobacter*-ის დათრგუნვის შესაძლო ტექნოლოგიად ჩამოყალიბების პილოტური ტესტირება, შემუშავებული ბიოტექნოლოგიისა და სამეცნიერო იდეების პატენტირება და საბოლოო ჯამში დაინტერესებული ორგანიზაციებისთვის კონკრეტული კომერციული რეკომენდაციების გავრცელება.

მიღებული შედეგები მნიშვნელოვანი წვლილის შემტანი იქნება კომპლექსური პრობიოტიკული პრეპარატის შემუშავებისთვის, რაც, თავის მხრივ, პრობიოტიკული ბაქტერიული შტამების მიერ *Campylobacter*-ის ბაქტერიოცინული მექანიზმით დათრგუნვას დაეფუძნება. აღნიშნული ტიპის ბიოპრეპარატები არა მხოლოდ უსაფრთხოა ფრინველებისათვის, არამედ მათ შეუძლიათ მოახდინონ კომპლექსური გავლენა ფრინველის იმუნური სისტემაზე, კერძოდ მათ უნარზე მეტად ეფექტურად ებრძოლონ სხვადასხვა ბაქტერიულ პათოგენს.

დისერტაციის შემდგომი განვითარებისა და გამოყენების პერსპექტივა

დისერტაციის ფარგლებში ჩატარებულ კვლევას აქვს სამომავლო განვითარების პერსპექტივა, რაც შესაძლებელია მოიცავდეს პრობიოტიკული შტამის მიერ პროდუცირებული ბაქტერიოცინის გამოყოფასა და ბიოქიმიურ დახასიათებას, *Campylobacter*-ის შტამების გამოყენებას in vivo ექსპერიმენტებში. ასევე, შემდგომ ეტაპზე სასურველია იმ პირობების დადგენა, რომელშიც ბაქტერიოცინი პროდუცირდება მაქსიმალური რაოდენობით. გამოყოფილი და გასუთავებული ბაქტერიოცინის, როგორც დამოუკიდებელი პროდუქტის, გამოყენება შესაძლებელია ნედლი ქათმის ტან-ხორცზე. მსგავსი პროდუქტები დაშვებულია, მაგალითად, აშშ-ს წამლებისა და საკვების სააგენტოს (The US FDA) მიერ ხორცში სხვადასხვა პათოგენის (მაგ. *Listeria*, *Salmonella* spp.) კონტროლისთვის. მნიშვნელოვანია აღინიშნოს, რომ

პათოგენების უსაფრთხო კონტროლის მექანიზმების შემუშავება ახალი ტექნოლოგიების განვითარებასაც გულისხმობს.

SAFS-ის სადოქტორო კვლევის ფარგლებში გამოქვეყნებულია სამი სტატია:

მიმოხილვითი სტატია:

Animals (MDPI), Feb 2020

"Application of *Campylobacter jejuni* Phages: Challenges and Perspectives"

Leonid Ushanov, Besarion Lasareishvili, Irakli Janashia and Andreas E. Zautner

ორი სტატია, რომელიც მოიცავს ექსპერიმენტული კვლევის შედეგებსა და მათ ანალიზს:

Annals of Agrarian Science, Nov. 2023

"*Campylobacter coli* and *Campylobacter jejuni* in Georgian Retail Chicken: Isolation, Identification and Antibiotic Susceptibility"

L. ushanov, I. Pipia, A. Bagishvili, A. Meshvildishvili, I. Abuseridze, N. Gagelidze

International Journal of Systematic and Evolutionary Microbiology

"*Arcobacter tbilisiensis* sp. nov. isolated from chicken meat in Tbilisi, Georgia"

Leonid Ushanov, Matthias Riediger, Annika Dreyer, Ia Pipia, Nino Gagelidze, Aljoscha Tersteegen,

Pauline Marquardt, Achim J. Kaash, Andreas E. Zautner.

1 Introduction

The aim of this PhD research is to assess the occurrence of *Campylobacter* and *Arcobacter* species in raw chicken meat sold in food stores and supermarkets in Tbilisi, Georgia. Another important goal is to generate data on phenotypic and genotypic characteristics of the *Arcobacter* isolates in order to assess their antimicrobial susceptibility and, based on the sequencing data, to evaluate the *Arcobacter* genomes for the presence of the genes encoding for virulence and antibiotic resistance factors. The bacterial strains involved in the present study were isolated over a two-year period between the fall 2018 and summer 2020.

The aforementioned goals were largely determined by the current circumstances in clinical and veterinary diagnostics in Georgia: to this date no data exist on the prevalence of *Campylobacter* spp. in raw chicken meat and other meat products in this country. Equally nonexistent are the data on the prevalence of *Campylobacter* spp. in the human population. There are two reasons for this: first, *Campylobacter* is not a reportable pathogen in Georgia and second, clinical laboratories in developing countries often fail to identify this infectious agent. This may be due to difficulties associated with isolation of *Campylobacter* spp. In fact, neither *Arcobacter* spp. nor *Campylobacter* spp. are part of routine diagnostics in developing countries. Thus, this PhD work is the first research that intends to close the gap in knowledge about the prevalence of *Campylobacter* spp. in locally produced raw chicken meat. This is especially important for bringing the Georgian public health up to the EU standards in the wake of receiving the EU candidacy status.

Broiler meat is commonly associated with *C. jejuni* and *C. coli* worldwide and continues to remain as the largest reservoir of *Campylobacter* spp., accounting for 50-80% of human infections. It has been estimated that 20–30% of human campylobacteriosis cases may also be due to improper handling. Research publications from Iran and Turkey indicated that *Campylobacter* and *Arcobacter* species were frequently isolated from chicken meat in these countries (Khoshbakht et al. 2014). Naturally, we assumed that local, Georgian, meat, especially raw chicken meat, would be contaminated with *Campylobacter* spp. at somewhat comparable rates, especially when there are no mechanisms in place in Georgia, to control

these microorganisms. Thus, the primary goal of this PhD research study became isolation and characterization of *Arcobacter* spp. and *Campylobacter* spp. from raw chicken meat produced and handled locally.

Before embarking on this PhD project, my interest was piqued by the mechanisms through which lactic acid bacteria (LAB) reportedly inhibit different Gram-negative pathogens, as highlighted in numerous publications. Scientific publications available on this topic often relied on data resulting from methods that could not be directly compared, thus requiring additional research and clarification. For example, most researchers implicated acidic pH resulting from the production of organic acids by LAB, such as lactic acid and acetic acid, and their accumlation in the surrounding medium. However, acidic pH could not always be correlated with the fact of inhibition. In other words, the same low pH value did not always cause inhibition of *C. jejuni* in two different studies. Therefore, another mechanism, which was being masked by the acidic pH, had to exist. It is a known fact that pH value below 4.5 eventually kills *Campylobacter* spp. Thus, bacteriocin-mediated inhibition of *Campylobacter spp.* became the second important goal of this PhD work. This naturally included isolation and testing of local LAB strains demonstrating such inhibitory qualities. Consequently, *Lactobacillus fermentum* was identified as the LAB strain that could effectively inhibit *Campylobacter* spp. and *Arcobacter* spp. in vitro at neutral pH, as demonstrated by the co-culture and cytotoxicity experiments. To further evaluate the efficacy of *L. fermentum* in vivo experiments need to be conducted in live birds sometime in future.

Finally, another important goal of the presented research was to evaluate newly isolated *Arcobacter* strains in terms of clonal relatedness, resistance to various antibiotics and presence of virulence factors.

2 Connection of the current research with the Sustainable Development Goals

2.1. What are the SDGs and why are they important?

In 2015, 193 member states of the United Nations (UN) agreed upon adopting the 2030 UN Agenda for Sustainable Development. The SDGs were designed to phase out Millennium Development Goals (MDGs) adopted earlier for the period 2000-2015 and were built on those goals. The Agenda encompasses 17 sustainable development goals associated with 169 targets (Asian Development Bank, Setboonsarng, and Gregorio 2017). These targets were designed to serve as inspirational goals for civil society, governments, various international organizations and private businesses, to plan and implement human development in the next 15 years. The Millennium Development Goals were not unsuccessful, but resulted in significant achievements during the 15 years of their implementation. Notably, eradication of extreme poverty and childhood mortality were the biggest achievements of the MDGs (Asian Development Bank, Setboonsarng, and Gregorio 2017). However, the MDGs also had shortcomings. For example, they were criticized for failing to address the interdependencies between the goals and for placing the responsibility on developing countries, instead of developing a universal approach for the entire world (Norström et al. 2014).

2.2. Sustainability in agriculture

In order to survive, humanity must urgently address the issues of sustainability, especially within the sphere of agriculture. The concept of sustainability was first conceptualized by the Brundlandt commission as the balance between the needs of the current and the future generations (Korthals, M. 2001). Sustainable agriculture implies that current generations may produce maximum amount of food, but without impacting negatively the ability of future generations to do the same. This means that food must be produced without harming the environment and upsetting the ecosystems and biodiversity. However, these goals will not be achievable without implementing certain measures. This is precisely what sustainable development goals are meant to address.

Sustainability and harmonization with the Sustainable Development Goals (SDG) are the cornerstone of this PhD thesis, which, besides identifying the unmet need-prevalence and characterization of *Campylobacters* in Georgian retail chicken-focuses on the challenge of controlling *Campylobacter* at the primary production level as well. This goal must be implemented with the consideration of the SDGs and it must take into consideration environmental, as well as social challenges. The conclusions and recommendations provided in this PhD work have a great potential of being integrated into sustainable development goals on multiple levels and have synergistic relationship across multiple indicators. Measures aimed at the reduction of *Campylobacter* in poultry farms through administration of an effective probiotic formula in the chicken feed would have beneficial effect on the health of farm-raised poultry, while consumers would benefit first-hand from the reduction of *Campylobacters* and *Arcobacters* by being exposed to less risk of contracting campylobacteriosis. Probiotic supplements could potentially decrease the use of antibiotics at farms due to their immunomodulatory effect and this could have positive environmental impact as well.

Achieving such ambitious goal, however, will require more activities, such as research and development efforts for creating of probiotic formulas, as well as business activities and marketing for the integration of the potential startup into the agricultural industry. Such activities would spur job-creation and help the local economy improve.

2.3. SDGs and reduction of *Campylobacter* spp. in farm-raised poultry

Based on the review of numerous publications on *Campylobacter* spp., specifically *C. jejuni* and *C. coli*- the two most clinically relevant species involved in human disease-there is an unequivocal connection between the human campylobacteriosis and the prevalence of *Campylobacter* spp. in poultry, specifically farm-raised broilers. An important factor in the goal of elimination, or at least decreasing, of *Campylobacter* spp. in primary production, has been the recognition of poultry farms as predominant reservoirs of *Campylobacter* spp. To date, elimination attempts of these microorganisms using preventive biosecurity measures and vaccinations have met with little success due to the fact that *Campylobacter* spp. are

ubiquitous in nature. Vaccination of broilers against *C. jejuni* have had limited effect, whereas, the effect of alternative methods of reduction, such as phage therapy and the use of probiotics have been moderate. On the other hand, accumulating evidence suggests that successful inhibition of pathogenic bacteria depends on a particular type of the pathogen and a particular strain of a probiotic. Additionally, when it comes to elimination of *Campylobacter* spp., factors like the administered dose of probiotics and phages, and the mode of administration make a significant difference.

Several studies reported significant (1-2 logs) reduction of *C. jejuni* in farm-raised poultry following application of bacterial cocktails formulated from various probiotic strains of lactobacilli and bifidobacteria. Probiotics given to chickens through feed, or water, help regulate the birds' immune response to various antigens by interacting with different subsets of cells of their adaptive immune system and stimulating the production of cytokines (Haghighi et al., 2005). Healthy immune systems and general wellbeing of birds should potentially decrease the use of antibiotics in order to control bacterial pathogens in the flocks. Decreasing the circulation of antibiotics in poultry farms is an important aspect in integrating the primary production into sustainable farming and the implementation of SDGs. However, we have to first revisit the SDGs and their history to clarify this point.

2.4. 2030 Agenda of sustainable development

The 17 SDGs differ from the previously developed MDGs in the sense that they cover much broader, universal issues, as they were designed to be applicable to all nations with the consideration that resources, such as land, water, energy and food, are limited. In this agenda, the concept of sustainability was broadened. For example, it was acknowledged that resources are limited and that, in supporting ecosystems and to develop further, environmental issues must be integrated into the concept of sustainability. Additionally, the concept of universality of the SDGs was based on the integration of three indivisible dimensions: economic, social and environmental. Thus, to achieve any goal, it must incorporate all three dimensions with no particular aspect having priority over another, i.e.

any particular goal must produce a result that integrates development across all three dimensions (Asian Development Bank, Setboonsarng, and Gregorio 2017).

The UN member states that adopted 2030 Agenda and Sustainable Development Goals should, naturally, demonstrate their commitment to the latter. A fine example of such commitment is reflected in the 2017 Foreign Policy White Paper of the government of Australia. Australia has committed to implement water reforms and improve the efficiency use of water resources and managing and improving freshwater ecosystems (Inquiry into the United Nations Sustainable Development Goals (SDGs)., 2017) Australian Government. Department of Agriculture and Water Resources, 2018). One of the goals of the government of Australia is to better regulate the farm business to promote healthier market (Australian Government. Department of Agriculture and Water Resources, 2018). Most importantly, Australia has committed to "farming smarter", i.e. base the increasing of the country's farming productivity on research and development and doubling both the agricultural productivity and the income of small farm producers.

In contrast to Australian efforts, the Government of Georgia has been focusing on different, more socially oriented priorities, such as improving social justice and economic well-being of its citizens. Significant achievements were made in Georgian healthcare. For example, the hepatitis C virus (HCV) elimination program cured thousands of people, achieving complete viral clearance and dramatically improving the quality of life for the enlisted patients. However, Georgia has been less active in addressing the issues related to sustainable farming and the climate change: the three dimensions were hardly ever mentioned in the official 2015 report on SDGs of the office of the former Prime Minister Giorgi Kvirikashvili (Office of the Prime Minister of Georgia 2016). Another report on SDGs produced by the Government of Georgia discussed SDG 16 exclusively (Tutberidze, M., IDFI, 2017).

2.5. Connection of this research with SDGs

2.5.1 Integrated approach to sustainability

Sustainable Development Goals were designed to transform the world, so that the major milestones, such as human wellbeing, economic prosperity and environmental protection, are achieved in an outcome that is mutually inclusive for all the goals. This means that the goals must be in harmony with each other, i.e. the interdependence of the goals has to be characterized with positive correlation. The multidimensional approach to sustainability recognizes all relevant dimensions within and across development goals and their implicit interdependent nature. The social discourse has become one of the focal points of the integrated approach and the latest version of the concept of sustainability takes into consideration the complex interconnections between the environment, economy and society. Pradhan et al. in their analysis of data on 122 indicators for a total of 227 countries for the years 1983-2016 available through the United Nations Statistics Division, demonstrated that there were significantly more synergies than trade-offs within each SDG. For example, SDGs 1 (No poverty), 3 (Good health and wellbeing), 4 (Quality education), 10 (Reduced inequalities), 12 (Responsible consumption and production), and 13 (Climate action) all demonstrated synergetic relations, i.e. the correlation values were greater than 0.6 for 80%–90% of the data pairs. The data also showed that the indicators were compatible and progress, for example, in one indicator was in harmony with the fulfillment other indicators within the same goal (Pradhan et al. 2017).

2.5.2 SDG 2: Zero hunger

Reduction of *Campylobacters* circulating within poultry farms could help maintain healthy flocks and, eventually, healthier, uncontaminated meat. This is directly connected with SDG 2, Zero Hunger. Developing countries, such as Georgia, need protein for healthy nutrition. Chicken meat and eggs are significant sources of protein and cheaper, compared to other sources, e.g. beef and pork. In developed and developing countries alike, chicken remains a popular food item. However, there are parts of the developing world where chicken meat

and even eggs are considered a luxury. Sub-Saharan Africa and South East Asia are particularly vulnerable to malnutrition and undernutrition, which are connected to poverty (Farrell, 2016). The nutritional value of chicken eggs is already high, however it is easy to make it even more nutritional. Producing chicken meat is not difficult and requires only 1.7 kg of feed per kilogram of chicken (Farrell 2016). The situation with hunger has improved in Georgia considerably in recent years, according to the 2016 report of Asian Development Bank. However about 10% of children still remain malnourished. Overall, compared to the 1990s and early 2000s, when Georgia was experiencing severe food shortages following the economic collapse, the situation has improved dramatically. Yet, there is still much to be done. Georgia has the capacity to increase production of poultry meat, because, according to the data produced by European Neighborhood Programme for Agriculture and Rural Development, only 20% of the total demand on chicken meat was satisfied by locally produced poultry (ENPARD, 2015). This means that Georgia can not only meet this demand, but also exceed it.

2.5.3 SDG 3: Good health and wellbeing

When we talk about health and wellbeing, we almost exclusively imply human condition. Animal wellbeing and environmentally responsible farming, arguably, can never be reconciled, due to the absence of basic freedoms that animals must enjoy for compassionate treatment (Korthals, M. 2001). Although the prevalent opinion is that *C. jejuni* does not seem to have any pathogenic effect in poultry and is a mere commensal organism in the intestinal tract of chickens, there are studies that prove the opposite: *Campylobacter* infections have been shown to be associated with chick mortality, while in laying hens *Campylobacters* have been associated with liver lesions (DaMassa A. J. 1992).

Production of *Campylobacter*-free meat will have a direct beneficial impact on public health and economy. For example, if in a healthy human *Campylobacter* infection lasts a few days in children and the elderly it may have a more complicated form. Recurrent infections and infections with antimicrobial resistant strains are possible in immunocompromised patients. *C. jejuni* may trigger Guillain-Barre syndrome (GBS) or Reiters syndrome (Udaya

Seneviratne 2000). 40% of patients with GBS can be traced to a recent *C. jejuni* infection (J. E. Moore 2002), (Ronner et al., 2004). One of the most important aspects of chicken meat production is the issue of antibiotics being used in farms, as well as emergence of resistant strains of different bacterial species.

2.5.4 SDG 8: Decent work and economic growth

Innovation and Infrastructure implies that Georgian meat producers, to stay competitive, must sooner or later introduce preventive measures to control *Campylobacter* spp. and *Arcobacter* spp. in their produce. This means that there is an opportunity to educate farmers and enable them to select the feed and other products for their farms based on knowledge and best practice. On the other hand, selecting and testing of new probiotic strains to create a probiotic formula able to inhibit *Campylobacter* spp. and other pathogens in the chicken gut would require attracting investments and launching a small production. This, in turn, would lead creating new jobs and stimulating the local economy. At this stage, however, the priority is to identify the strains of probiotics and test their in vivo activities.

2.5.5 SDG 9: Sustainable infrastructure and innovation

Integration of new technologies, ideas and approaches into the attempts to solve existing problems, or address the potential ones are crucial to innovation. A locally developed probiotic formula intended to maintain the gastrointestinal health of the farm-raised poultry, would be the first such environmentally friendly product to control pathogenic microorganisms like *Campylobacter* spp. *and Arcobacter* spp. This would inevitably contribute to innovation in the meat industry. This can only happen if both science and business aspects were integrated into a single, streamlined approach. For example, testing the effect of such product would require in vivo efficacy studies, which would in turn create the need of seeking collaboration with the industry to conduct such experiments. Improvements and adjustments to the composition of the formula, such as integration of new prebiotic microorganisms and other additives able to enhance the efficacy and stability of the probiotic cocktail, would require additional studies and financing. However, all these aspects are a

consideration for the future. The immediate goal at hand is to investigate the mechanism of action of the current leading probiotic candidate, which is *L. fermentum.*

Globally, *Campylobacter* is associated with significant threat to public health in both the developed and the developing worlds. This human pathogen has not been studied in Georgia since 1970s and currently no governmental agency is monitoring *Campylobacter* spp.. Even for countries with advanced economies, elimination of *C. jejuni* has proven to be challenging: this effort requires complex measures, such as improved sanitation, providing clean water and environment for the farm-raised flocks along with strict biosecurity measures introduced in hatcheries and throughout the entire farm. Incorporation of specific and effective probiotic cocktail in chicken feed is one way to help decrease *Campylobacter* spp. in chickens. This approach is not only environmentally friendly, but may also have many other benefits. The benefits resulting from the elimination of *Campylobacter* spp. in farm-raised poultry go beyond the concepts of health and healthy food and fit very well into 2030 UN Agenda for Sustainable Development Goals. The indicators associated with such benefits may be synergistic with multiple other indicators.

There are a few medium and many small-scale, household-managed poultry farms in Georgia that produce retail chicken. Among the largest producers "Chirina" stands out due to its ability to implement vertical integration. Using Israeli management and innovative technologies in animal nutrition, this company was able to create notable synergies that noticeably drove down the market price of chicken meat since 2013, when the company was first established (Livni, USAID Georgia, 2014). To the best of our knowledge, however, neither "Chirina", nor any other manufacturer of poultry meat, monitors *Campylobacter* spp. in their primary production. Unless monitoring *Campylobacter* spp. load is not required by the State, companies are not going to bear the expense and support any measures involving testing and monitoring of *Campylobacter* spp. in their produce. This may change, however. Georgia and European Union signed the association agreement and the Deep and Comprehensive Free trade Agreement (DCFTA) in 2014 and now efforts are being made, from both sides, for a better integration of Georgia into the EU trading space (DCFTA

European Union Legislation 2014). Signing this agreement explicitly states that the goal of the agreement is "to promote political association and economic integration between the parties based on common values and close links, including by increasing Georgia's participation in EU policies, programmes and agencies"[1]. The association agreement implies transposition of EU's legal standards into Georgia's legislative system (Office of the Prime Minister of Georgia 2016, First National Voluntary Review of SDGs). From January 1st,, 2018, testing for *Campylobacters* became compulsory for the meat producers and the limit of detection constitutes 1000 CFU/g[2]. The sooner Georgia follows the lead, the better. This is going to be a good development in Georgian public health policy.

3 Literature Review

3.1. *Campylobacter jejuni* and *Campylobacter coli*

Campylobacter spp. are short and fine, curved, motile, microaerobic Gram-negative rods common to many different animal hosts including livestock, pets and wild animals (Battersby et al., 2016; Corcionivoschi et al., 2015). The discovery of this bacterium is attributed to Theodor Escherich, who was the first to identify the spiral-like rods in stained mucous samples from children and kittens with diarrhea, under the light microscope (Kist, 1986). The microorganism became known under various names, including *Vibrio coli.* Although Escherich was not able to culture the pathogen on solid medium, in 1970s scientists managed to isolate *Campylobacter jejuni-a* recognized human gastrointestinal pathogen known today (Butzler, 2004). The most clinically important and frequently isolated *Campylobacter* spp. associated with human disease are *C. jejuni*, *C. coli, C. lari* and *C. upsaliensis,* while *C. fetus* appears to be a major veterinary pathogen (Igwaran and Okoh, 2019).

While animals, including cattle and domestic pets, may carry *Campylobacter* spp. asymptomatically, humans, after exposure to this pathogen, develop mild or severe bloody diarrhea that may or may not be accompanied by fever and cramps (Khoshbakht, et al., 2014; Johnson et al., 2017). Although the majority of human Campylobacter infections are self-

1 https://www.eeas.europa.eu/delegations/georgia/eugeorgia-association-agreement_en
2 https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32017R1495&rid=1

limiting, antibiotics may become necessary in severe cases, in immunocompromised patients, or when treating prolonged infections, especially in children (Mattheus et al., 2012).Today *C. jejuni* and *C. coli* are the most frequently encountered *Campylobacter* spp. out of total 39 known described to date (M. F. Silva et al., 2020; Zenebe et al., 2020). In humans, *C. jejuni* is responsible for 80-90% of infections, while 5-10% of human *Campylobacteriosis* cases are caused by *C. coli and* other *Campylobacter* species (Davies et al., 2020). The most important post infection sequelae caused by *Campylobacter* spp. is the Guillain-Barré syndrome-an acute neuropathy that results in demyelination of peripheral nerves and paralysis (Hemal et al., 2016).

According to numerous studies, *Campylobacter* spp. are considered harmless commensals in chicken. However, some studies indicate that this might not be generally true. For example, research on the prevalence of *Campylobacter* in laying hens identified that both *C. jejuni* and *C. coli* might be associated with vibrionic hepatitis with characteristic lesions forming in chicken livers (DaMassa A. J., 1992). Studies in laying hens are in fact rare due to a very low risk of transmission of Campylobacter spp. through eggs and almost nonexistent vertical transmission of the pathogen. Campylobacter spp. have been identified not only in the intestines of the laying hens, but extra intestinally as well: for example, in the liver and the spleen. At times, both *C. coli* and *C. jejuni* were isolated from one individual hen, which agrees with our findings (DaMassa A. J.,1992).

Occurrence of Campylobacter-related enterocolitis in the world, according to various sources, amounts to 400-500 million cases yearly (Vlieghe et al., 2008). It has also been estimated that worldwide 50% of chicken meat is contaminated with *Campylobacter* spp. (Vandeputte et al., 2019). Among all known *Campylobacte*r species *C. jejuni* is considered the leading cause of human campylobacteriosis in industrialized countries. The majority (about 90%) of the food-borne illnesses caused by *Campylobacter* spp. are associated with *C. jejuni*, whereas, about 5-10% of the cases are attributed to *C. coli* and *C. lari,* however differences in the prevalence of certain isolates vary geographically (Igwaran and Okoh, 2019). Biochemically *Campylobacter* spp. are relatively inert: they poorly hydrolyze sugars

and, to distinguish between species, scientists rely on a few biochemical characteristics (Burnett et al., 2002). To differentiate *C. jejuni* from other *Campylobacter* spp. microbiologists use its ability to hydrolyze hippurate. However, about 10% of *C. jejuni* isolates are unable to hydrolyze hippurate. Moreover, there is another, hippuricase-positive species-*C. avium*-first isolated in Italy in 2006 from chicken (Miller et al., 2017). Therefore, molecular or genomics-based methods should be used to effectively distinguish between these two species of *Campylobacter*.

Clinical isolates of *Campylobacter* spp. are mostly of *C. jejuni* and *C. coli* of many sub-types, as demonstrated by PFGE (Pulse Field Gel Electrophoresis) fingerprinting and serotyping (Sheppard and Maiden, 2015). MLST (Multilocus Sequence Typing) is another extremely useful tool for genotyping and source attribution of *Campylobacter* isolates. With the help of this tool *C. jejuni* and *C. coli* can be differentiated and grouped as either clinical, or animal isolates. For example, the population structure of *C. jejuni* is highly diverse and MLST results may exceed 9000 in sequence types (STs), divided into several different clonal complexes (CCs). Additionally, *C. jejuni* CCs may consist of either host/niche-specific "specialists", or "generalists" that nonspecifically colonize various hosts. In contrast, the *C. coli* population is divided into three related clades. Therefore, the three clades of *C. coli* are linked to specific ecological niches. Typically, isolates from clinical and farm animal samples are primarily found in clade 1, while environmental isolates are categorized into clades 2 and/or 3 (Sheppard and Maiden, 2015).

3.2. Worldwide Distribution of *Campylobacter* spp. and *Arcobacter* spp.

3.2.1 *Campylobacter* spp. in the United States and Canada

Just five years ago *Campylobacter* spp. ranked the third among food-borne bacterial illnesses in the United States after Salmonella spp. and *Clostridium perfringens,* according to scientific publications (Marasini et al., 2018). Recently *Campylobacter* spp. have been cited as number one in incidence in the US. For example, out of 22,019 infections identified in the US, the incidence was highest for *Campylobacter* spp. (17.8 cases per 100,000 population) followed

by *Salmonella* (14.2 cases per 100,000 population). It is important to note that this change was not due to an increase in the *Campylobacter* incidence. Instead, *Salmonella* incidence in the US has decreased (Collins et al., 2022). According to USDA data for the fourth quarter of the fiscal year 2021, the presence of *Campylobacter* spp. in chicken meat ranged from 22.19% to 73.33%. In the United States, the Food Network identified 6,621 cases of *Campylobacter* infections in 2020, yielding an incidence of 13.82 per 100,000 people.

In Canada, campylobacteriosis was added to the list of reportable diseases in 1986. According to the data downloadable from the Public Health Agency of Canada's webpage, the incidence of *Campylobacter*-related illness increased from 27.23 per 100,000 people in 1991 to 43.58 per 100, 000 people in 2021. As in the US, *Campylobacter* spp. are the leading cause of food-borne bacterial infections in humans in Canada as well, chicken meat being one of the most common sources of human campylobacteriosis in this country (Hodges et al., 2019).

3.2.2 *Campylobacter* spp. in Australia and New Zealand

Campylobacteriosis is a nationally notifiable disease in Australia and *Campylobacter* spp. are the most commonly reported cause of food-borne human gastroenteritis in this country. More than 37,000 of *Campylobacter* infection notifications were reported in Australia in 2021 (Australian Institute of Health and Welfare, 2022). At the inception of the Australia's National Notifiable Diseases Surveillance System in 1991 the incidence of campylobacteriosis was 79.1 per 100,000 population and rose to 139.7 per 100,000 population by 2015. The incidence of Campylobacteriosis in New Zealand is somewhat similar to that of Australia. For example, in 2014 it was 150.3 per 100,000 population.

Campylobacter notification rates in Australia and New Zealand are higher compared to other high-income countries. For comparison, the reporting rate in the EU is 100 per 100,000 population (Varrone et al., 2018).

3.2.3 *Campylobacter* spp. in the European Union

In 2020 Campylobacteriosis in the EU moved to the first position exceeding the reported cases of salmonellosis thus making *Campylobacter* the most commonly reported

gastrointestinal bacterial pathogen in humans (European Food Safety Authority and European Centre for Disease Prevention and Control 2021). The notification rate in the EU was 55 per 100,000 people in 2020. Poultry, including broilers, laying hens, turkeys and ducks, account for 50% to 70% of human *Campylobacter* infections (Epps et al., 2013). According to EFSA, over 246,000 cases of Campylobacteriosis occur annually in the EU, although there are estimates that the actual number may be several fold greater (European Food Safety Authority and European Centre for Disease Prevention and Control, 2021). The incidence of *Campylobacter*-related illness varies geographically within the EU. For example, *Campylobacter* enteritis was cited as the second most common bacterial food-borne infectious disease in Slovenia, where the annual reported incidence of campylobacteriosis increased by 1.3% from 53 to 67.3 cases per 100,000 inhabitants (Zorman et al., 2006), while in Belgium, where campylobacteriosis became a leading zoonotic infection since 2005 with subsequent fluctuations, the annual reported incidence jumped from 49.9 to 73.6 per 100,000 inhabitants in the same time period (Mattheus et al., 2012).

Generally, *Campylobacter* infections have been on the rise during the past two decades in both the developed and developing countries in parallel with resistance of *Campylobacter* species to various antibiotics (Agunos et al., 2014).

3.2.4 *Campylobacter* spp. in the rest of the world

Studies from China, Brazil, India, Iran, Turkey and African countries, such as South Africa, Senegal and Ghana, all report high rates of *Campylobacter* isolates from chicken and other meats, indicating worldwide distribution of this pathogen. So is its increasing resistance to various antibiotics. For example, an Iranian study of 2019 identified high levels of resistance among *C. jejuni* and *C. coli* isolates from children with bacterial enteritis. Resistance to ampicillin constituted 100% and 90% among *C. jejuni* and *C. coli* isolates, respectively. At the same time, 20% of all strains were resistant to gentamicin (Ghorbanalizadgan et al., 2019). Painstil et al. identified *C. coli* and *C. jejuni* as the most frequently isolated enteric pathogens both from human and animal samples in West Africa (Paintsil et al., 2022). Brazilian researchers reported 90% prevalence of *C. jejuni* among chicken isolates (Würfel et

al., 2019). Thai researchers identified high incidence of MDR strains among both *C. jejuni* and *C. coli* from chicken isolates of *Campylobacter* (Thomrongsuwannakij et al., 2017).

3.2.5 *Campylobacter* spp. in broilers: the role of chickens as the host

Numerous publications implicate raw chicken meat as the primary source of *Campylobacter* infections. Source attribution studies performed on multilocus sequence typing data concluded that chicken meat is the main reservoir of *Campylobacter* spp. and a significant source of human campylobacteriosis cases (Haas, Overesch, and Kuhnert, 2017). For example, a recent Canadian study conducted in 2019 in Nova Scotia isolated *Campylobacter* spp. from chicken meat and clinical samples taken from diseased humans with the aim to evaluate any existing correlation between clinical isolates of *Campylobacter* spp. with those recovered from chicken meat. Comparative Genomic Fingerprinting analysis revealed that 36 subtypes common between the subtypes isolated from chicken (n=99) and those isolated from clinical samples (n=153) represented 48.3% of all clinical isolates. This unequivocally confirmed retail poultry as the largest reservoir of the *Campylobacter* subtypes (Hodges et al., 2019). In 2014 researchers from Tulsa University (Tulsa, OK, USA) determined that most of the STIs isolates of *C. jejuni* that came from various meat sources, including chicken gizzards and chicken livers, were the same as those isolated from diseased humans (Noormohamed and Fakhr, 2014). A Danish research group that investigated the distribution of serotypes of *C. jejuni* and *C. coli* among the strains isolated from chicken and beef and humans, concluded that the prevalent *Campylobacter* species in chicken and beef was *C. jejuni* (83-91%), while 95% of the isolates from pork were speciated as *C. coli.* Among the human isolates, the majority (95%) were reported as *C. jejuni* and only 6% belonged to *C. coli*, while 62% of the *C. jejuni* isolates were serotyped as O:1, O:2 and the O:4. These same serotypes were commonly identified in *C. jejuni* isolates obtained from broilers and cattle (Nielsen, Engberg, and Madsen, 1997). A source attribution study based on MLST genotypes from poultry (n=139), cattle (n=48), wild birds (n=101) and human *C. jejuni* infections (n=132) was conducted in 2020 across the Baltic states on 420 human and animal isolates of

C. jejuni. Follow up analysis identified poultry (88.3%) as the main source of *C. jejuni* in human infections (Mäesaar et al., 2020).

Prevalence of *Campylobacter* in chicken meat varies considerably between different geographic regions. For example, in some countries, such as Canada*, C. jejuni dramatically* outnumbered *C. coli* among *Campylobacter* spp*.* among the chicken isolates: 87% vs. 12%, according to a 2019 Canadian study (Dramé et al., 2020). A Brazilian study conducted in the same year identified that 87% of the total *Campylobacter*-positive samples resulted in the isolation of *C. jejuni* (Rodrigues et al., 2021). In other countries, however, *C. coli* was isolated at the rates equal to, or exceeding those of *C. jejuni*. For example, a recent Australian study found that the majority of *Campylobacter* isolates from fresh and frozen chicken carcasses and meat belonged to *C. coli* (50-77%) whereas, *C. jejuni* isolates were more common in beef, pork and lamb (50-88%) (Walker et al., 2019). A 2020 Chinese research determined that among the 464 isolates positive for *Campylobacter* spp*., C. jejuni* and *C. coli were* identified roughly at equal numbers-233 and 231, respectively (Tang et al., 2020). An Italian study from 2016 identified more *C. coli* than *C. jejuni among* their campylobacter isolates (91 C. coli and 41 C. *jejuni* from a total of 140 samples that included cloacal swabs and neck skins) (Pergola et al., 2017). A higher percentage (75.5%) of *C. coli* among the *Campylobacter* isolates from chicken meat compared to *C. jejuni* (24.5%) was also identified in a 2011-2013 Polish study (Szczepańska et al., 2015a). However, a later (2017) study, also conducted in Poland, concluded that *C. jejuni*, not *C. coli*, was the predominant *Campylobacter* species in poultry meat (Szosland-Fałtyn et al., 2018). Subsequent Polish studies reported once more the predominance of *C. coli* over *C. jejuni* among chicken isolates along with high incidence (70%) of resistance to ciprofloxacin (Wieczorek, Bocian, and Osek, 2020). A similar 2015 study from Thailand also identified more *C. coli* (n=94) than *C. jejuni* (n=36) among the isolates obtained from samples taken in and around chicken farms and hatcheries (Thomrongsuwannakij et al., 2017). A 2011-2013 Argentinian study investigated prevalence of *Campylobacter* species in abattoirs and retail chicken meat (including kosher meat), in and around Buenos Aires. Here, C. *jejuni* outnumbered *C. coli*

both in kosher (36% to 2%) and conventional (26% to 4%) meat. This finding agreed with previous Argentinian studies where *C. jejuni* isolates significantly outnumbered those of *C. coli* (Guirin et al., 2020).

The source of *C. coli* in chicken meat could be explained by contamination. For example, Zorman et al. identified significant number of *C. coli* among retail chicken isolates and close similarity between the isolates indicated cross-contamination during the processing of chicken carcasses (Zorman et al., 2006). In their research Guirin et al. identified a statistically significant difference between the killing methods used and *Campylobacter* spp. isolated. Specifically, the killing method may play a role not only in the level of contamination, but also the proportion of the contaminant *Campylobacter* species. This research also determined that in individuals infected with both *C. coli* and *C. jejuni* the two species may be differentially distributed in the chicken body. For example, *C. coli* tend to be localized in the liver, while *C. jejuni* are mostly found in the ceca and the intestines (Guirin et al., 2020).

According to the scientific opinion that prevailed in the past, diversity within the two species of *Campylobacter-C. Coli* and *C. jejuni*-resulted from their presence in the wild populations of birds and animals. However, increasing evidence suggests that anthropogenic factors could be the driving force in the evolution of these microorganisms (Sheppard and Maiden, 2015). The unprecedented growth of human population has been accompanied with increasing demand for food and intensification of agriculture and livestock farming. Industrial farms, characterized by their large populations of broiler chickens, have created a new environment for Campylobacter species. This has led to the emergence of *C. jejuni* lineages that are capable of infecting multiple hosts, as well as an expansion of a specific *C. coli* lineage found in both agricultural animals and diseased humans. Additionally, the prevalence of resistant lineages of both *C. jejuni* and *C. coli* has increased, with genetic exchange occurring between these lineages (Sheppard and Maiden, 2015). Studies have also identified differences in *Campylobacter* infections in rural and urban areas: the largest reservoir contributing to human *Campylobacter* infections in urban areas is poultry, whereas

this is not necessarily true in rural settings where other modes of transmission gain significance (Skarp et al., 2016).

3.3. *Campylobacter* spp. in the environment

3.3.1 *Campylobacter* spp. in wild birds

Numerous data suggest that consumption of inadequately cooked chicken meat and/or unpasteurized milk, is not the only route of transmission of *Campylobacter* spp., there are also accounts of campylobacteriosis outbreaks due to contaminated drinking water (Muller, Böhland, and Methner, 2011). Water has been recognized as a significant source of *Campylobacter* transmission. However, wild birds also serve as a reservoir of *Campylobacter* spp. Contamination events may occur due to proximity of wells with drinking water to sewage or as a result of leakage of animal fecal matter into drinking water reservoirs. Additionally, sewage effluents and agricultural runoffs from farms may end up in drinking water during heavy rainfalls (Trigui et al., 2015).

Scientists agree that colonization of animals, including chickens, is environmentally driven and water plays the key role in spreading of *Campylobacter* spp. within and between flocks of birds. For example, a Swedish research group determined that *Campylobacter* spp. are found in high numbers among ground foraging wild birds and opportunistic feeder birds. In this research, among the environmental sources, water was identified as the most important element that may harbor *Campylobacter* spp. (Waldenström et al., 2002). Worldwide, *Campylobacter* spp. are highly prevalent in both domestic and wild birds. For example, several studies demonstrated that both *C. jejuni* and *C. coli* are prevalent among waterfowl, such as common teal (76%). Out of 53 total isolates in this study, 75% belonged to *C. jejuni*, while 25% belonged to *C. coli* (Gargiulo et al., 2011). A 2014 Japanese study identified a 20% prevalence of *Campylobacter* spp. in different wild birds. In this case too, the majority of isolates belonged to *C. jejuni* (Shyaka et al., 2015). A Danish study conducted in 2016 found a correlation between the occurrence of *Campylobacter* spp. in Danish cattle and chicken

farms and the prevalence of *C. jejuni* in wild birds-blackbirds and sparrows. For example, 62% and 21% of blackbirds and sparrows, respectively, sampled on a total of 12 farms, were carriers of *C. jejuni* (Hald et al., 2015). A large study conducted in Korea throughout 2009 and 2010 sampled over 2000 birds, including migratory species, and identified 15% prevalence of *Campylobacter* species with no signs of pathogenicity (Kwon et al., 2017). Even captive animals, such as cynomolgus monkeys, may also carry both *C. jejuni* and *C. coli*, including MDR strains of both species, with or without clinical symptoms (Koga et al., 2017). Numerous research publications from across the world report isolation of *Campylobacter* spp. from various species of wild birds, including birds of prey, waterfowl, crows and pigeons, which proves the global presence of these pathogens in the environment. Thus, wild birds are a significant and recognized environmental source of *Campylobacter* (Ahmed and Gulhan, 2022).

3.3.2 *Campylobacter* spp. in the environmental waters

Some *C. jejuni* strains have been reported to survive for weeks in the environmental waters. The length of survival of these strains may depend on the existing specifics of a particular strain (Mughini-Gras et al., 2016). Transmission of *Campylobacter* spp. through water may take place directly by ingesting contaminated water, or indirectly via recreational use of lakes and ponds polluted with the feces of wild birds. Natural and artificial water reservoirs where agricultural runoffs from farms with colonized livestock often end up, may serve as a significant source of contamination (Nilsson et al., 2018). In fact, *Campylobacter* spp. can be frequently isolated from surface waters and may serve as indicators of recent contamination of the waters with animal feces and/or agricultural runoffs. The role of the environment in the transmission of *Campylobacter* spp. can be exemplified by an occurrence during the 2003 avian flu epidemic in the Netherlands where a reduction in campylobacteriosis cases by 44-50% coincided with the massive culling of infected birds in the areas where the culling occurred, suggesting that the spread of *Campylobacter* spp. was largely environmentally driven (Mughini-Gras et al., 2016).

Several studies have demonstrated that survival of *Campylobacter* spp. in the environmental waters was temperature dependent. For example, at 4°C *Campylobacter* spp. can survive for several weeks in both seawater and freshwater, while survival time at temperatures closer to 25°C drops dramatically to several days, which means that the presence of *Campylobacter* spp. in environmental waters is driven by seasonality (Trigui et al., 2015; Jones, 2001). Some sources, such as US CDC, report that in the developed countries the diagnosed campylobacteriosis cases are frequently acquired as a result of travel to the developing world. However, there are significant reservoirs of *Campylobacter* species in the developed countries found in farm-raised chickens with high prevalence of *Campylobacter* spp., and even in wild birds that may serve as significant vectors of *Campylobacter* species (Szczepanska et al., 2017).

3.4. *Arcobacter* spp. as emerging pathogens

The genus *Arcobacter* belongs to the family of *Campylobacteraceae* of the epsilon division of Proteobacteria (Vandamme et al., 1991). The hypotheses about *Arcobacter* being an emerging human pathogen transmitted via food chain started to appear in early 1990s (Shah et al., 2011). *Arcobacter* spp. have recently been designated as emerging food-and waterborne pathogens by the World Health Organization, due to their increasing association with meat and meat products around the world (Son et al., 2007; Collado and Figueras, 2011). According to some research, *A. cryaerophilus* is the dominating species among other *Arcobacter* spp. found in wastewater and two subgroups of this species have been detected- 1A and 1B (Collado and Figueras, 2011; Pérez-Cataluña et al., 2018)). This differentiation is based on the 16 RNA-RFLP patterns, which some researchers do not find useful, indicating that the taxonomy of the *A. cryaerophilus* is not yet completed (Pérez-Cataluña et al., 2018). Miller et al. suggested a reclassification based on the elements of free-living bacteria, such as genes responsible for sulfur metabolism, found in *Arcobacter*'s genome (Miller et al., 2007). The subgroups of *A. cryaerophilus* may co-exist in one animal or food source, however subgroup 1B is more frequently isolated than 1A (Pérez-Cataluña et al., 2018). In some instances, both subgroups may co-dominate one niche and, in others, one subgroup's

domination may depend on ecological factors, such as water temperature (Pérez-Cataluña et al., 2018).

Both *A. cryaerophilus* and *A. butzleri* have been recognized as potential hazard to human health by the International Commission on Microbiological Specification for Foods (Collado and Figueras, 2011). However, the actual impact on human health is still not clear and is a subject of scientific debate (Brückner et al., 2020). Moreover, *Arcobacter* spp. have been isolated from clinically healthy humans in Switzerland (1.4% of the population tested) and South Africa (3% of the population tested) (Collado and Figueras, 2011; Shah et al., 2011).

The significance of the members of the genus *Arcobacter* as potential pathogens has been steadily growing since 1990-s when the first three species-*A. butzleri, A. cryaerophilus* and *A. skirowii*-were first classified. Today the genus *Arcobacter* comprises 30 members, including the novel species *A. tbilisiensis* sp. nov. identified in this study. This recent increase in the frequency of isolation of *Arcobacter* spp. could be ascribed to both improved media and isolation techniques. While acquisition of virulence factors has been proposed as one of the reasons behind the pathogenicity of *Arcobacter* spp., it seems more plausible that in the past *Arcobacter* infections were either misdiagnosed as *Campylobacter* infections, or the bacterium could not be cultured. Even today *Arcobacter* spp. are not part of the routine culture in clinical laboratories (Gonzalez et al., 2000).

The absence of standardized procedures of isolation may greatly affect *Arcobacter* incidence in human infections reported from countries around the world (Brückner et al., 2020). Apart from the impact of the methodology used, ecological differences and variations in sample sizes most probably affected the results of the studies as well (Brückner et al., 2020).

Microscopically *Arcobacter* spp. are almost indistinguishable from *Campylobacter* spp. Not surprisingly, these bacteria were previously classified as aerotolerant *Campylobacter-like* microorganisms, until in 1991, Vandamme and De Ley reclassified them into a separate genus within *Campylobacteraceae*, based on RNA and DNA hybridization and immunotyping experiments. *Arcobacter* spp. are very fine, slender and short Gram-negative rods with curvature characteristic of *Campylobacter* spp. This microorganism is nonsporeforming and

motile and can be cultured microaerobically or aerobically at temperatures between 15-37˚C (Vandamme et al., 1991). *Arcobacter* requires 2 to 5 days for growth. Aerotolerance and the ability to grow at low temperatures gives the members of the genus *Arcobacter* advantage to thrive in the environment. Biofilm formation is another adaptive trait of *Arcobacter* spp., which helps these microorganisms grow in different environmental niches, including abiotic surfaces, such as polyethylene, stainless steel and copper (Šilha et al., 2021).

Arcobacter spp., much like *Campylobacter* spp., carry putative virulence genes, as demonstrated by several studies (Douidah et al., 2012). Many of the virulence genes of *Arcobacter* spp. have been reported to be similar to those found in *Campylobacter* spp., e.g. *cadF* (adhesion factor) and *ciaB* (invasion factor). A 2013 Iranian study identified *cadF* and *ciaB* in all of 113 *A. butzleri* isolates using PCR, while in *A. cryaerophilus* isolates *cadF* and *ciaB* were found at 55% and 97% frequencies, respectively (Tabatabaei et al., 2014). A more recent study conducted in 2018 did not identify the adhesion factor *cadF* and *hecA* or *hecB* (adhesion protein and a factor F or hemolysis activation, respectively) based on whole genome sequencing of geographically and ecologically different *Arcobacter* isolates (Pérez-Cataluña et al., 2018). Significant heterogeneity was observed in a Japanese study of cytotoxic effect induced by *Arcobacter, including* cell elongation due to enterotoxin production, and adherence without invasion using VERO, CHO, HEP-2 and HELA cells (Carbone et al., 2003). Similar cytotoxic effects were shown by the retail meat isolates of *Arcobacter* spp. Ciprofloxacin has been reported by some studies as effective against *A. butzleri* and *A. cryaerophilus,* however, resistance may occur due to mutations in the *gyrA* gene/quinolone resistance determining region (QRDR) (Abdelbaqi et al., 2007). Resistance to ciprofloxacine was also observed among several isolates obtained in this study.

One major problem with the enumeration and evaluation of the prevalence of *Arcobacter* spp. in water, foods and other sources, is the absence of standardized methods of isolation and quantification of these microorganisms. Researchers who reported data on *Arcobacter* spp. often used methods that could not be compared. For example, quantitative PCR and conventional culture used for the quantification of *Arcobacter* spp. in various samples could

yield completely different results. Additionally, biochemical differentiation of *Arcobacter* spp. can be challenging as well, because, like in *Campylobacter* spp., identification *of Arcobacter* spp. is based on a few biochemical tests (Neubauer and Hess, 2006). Instead, to identify the exact species, researchers rely on DNA probes, PCR, and whole-cell protein SDS PAGE. PCR identification is especially useful due to the fast turnaround time and ability to avoid ambiguities of some biochemical methods (Atabay et al., 2003).

3.5. *Arcobacter* spp. in humans

A. cryaerophilus infections in humans result in acute enteritis with vomiting, watery or bloody diarrhea, abdominal pain and fever (Uljanovas et al., 2021). In most cases these infections resolve within a few days and do not require antibiotics. However, since *Arcobacter*-related bacteremia and peritonitis have been reported in immunocompromised patients, antibiotic-based therapy is recommended in this population. For example, following isolation of *A. butzleri* from a neonate with bacteremia acquired in-utero, a baby was administered antibiotic treatment consisting of intravenous penicillin and cefotaxime for 6 days (On, Stacey and Smith, 1995).

According to recent studies, *Arcobacter* was the second most frequently isolated microorganism from human fecal samples in Germany and the fourth most common bacterial isolate from human stool samples in Belgium (Shah et al., 2011) Studies from India, Thailand, Mexico and Guatemala show that *Arcobacter* spp. can be associated with traveller's diarrhea (Shah et al., 2011). In a 2014 Chilean study that used fecal samples of various origins, including children's, low prevalence of *Arcobacter* was identified in human feces, however higher prevalence was noted in fecal samples of bovine, porcine and chicken origin. As of today, no standards exist for the routine isolation of *Arcobacter* in clinical laboratory settings, making identification of this zoonotic pathogen challenging. For example, in a Costa Rican case study of a severe diarrhea in a female patient, the causative agent, *A. cryaerophilus*, was identified in an environmental and not a clinical, laboratory due to the absence of routine workup procedure for *Arcobacter* at the latter (Barboza et al., 2017). In 2013 an outbreak of *A. butzleri* was described for the first time in the attendees of a wedding

in Wisconsin as a result of consuming roasted chicken. Interestingly, all but one patients, whose stool sample tested positive for *A. cryaerophilus*, were infected with *A. butzleri* (Lappi et al., 2013). A German study identified *A. butzleri, A. lanthieri* and *A. cryaerophilus* among human isolates. 36 strains of these bacteria were isolates from more than 4000 samples processed. The isolates were characterized with high genetic diversity, while *A. cryaerophilus* appeared to be less virulent, compared to *A. butzleri* and *A. lanthieri* (Brückner et al., 2020).

3.6. *Arcobacter* spp. in chicken meat

Association of *Arcobacter* spp., particularly *A. butzleri* and *A. cryaerophilus*, with chicken meat has been well-demonstrated (Neubauer and Hess, 2006). According to some research, chicken meat allows for isolation of *Arcobacter* more frequently than any other meats (Shah et al., 2011),(Kabeya et al., 2004). Some researchers suggested that presence of *Arcobacter* spp. in chicken feces is in fact low and most contamination is originated from the processing equipment (Kjeldgaard et al., 2009).

Researchers from around the world have isolated both *A. bultzeri* and *A. cryaerophilus* from chicken carcasses. In a 2002 study conducted in Turkey, researchers sampled 75 (44 fresh and 31 frozen) chicken carcasses obtained from different markets. 95% of the fresh chickens and 23% of frozen chickens enabled isolation of *A. butzleri*, while no *A. cryaerophilus* was isolated (Atabay et al., 2003). Researchers from the Netherlands sampled 2 broiler and 3 chicken flocks in two chicken slaughterhouses. Samples were taken from supply water, one group of chicken feces and the chicken carcasses and viscera. This resulted in finding that most chicken carcasses and intestines were *Arcobacter*-positive, while no *Arcobacter* was detected in the supply water. Thus contamination might have originated during the slaughter (Ho et al., 2006). In another study conducted in Denmark, both *A. butzleri* and A. *cryaerophilus* were identified as chicken meat contaminants (Atabay et al., 2006). The Isolates from raw chicken meat in Costa Rica included *A. butzleri, A. cryaerophilus, A. thereius* and *A. skirowii* (Bogantes et al., 2015). A study in the United States sampled chicken carcasses in a commercial processing plant and identified high prevalence of *A. butzleri* and

A. cryaerophilus on pre-scold and pre-chill carcasses (97% and 61%, respectively). Post-chill carcasses yielded significantly less (9.6%) isolates. Out of total isolates *A. butzleri* (79%) prevailed followed by *A. cryaerophilus* (21%). Several other studies demonstrated the presence of *Arcobacter* species in poultry meat, such as chicken viscera (17.3%), chicken breasts (56%) and minced chicken meat (48%) (Son et al., 2007). An Italian study of 2011 identified that 39% of poultry meat samples were contaminated with *A. butzleri* exclusively (Amare et al., 2011). Similarly, a Malaysian study identified 39% of retail chicken meat contaminated with exclusively *A. butzleri* (Amare et al., 2011).

Retail chicken often accumulate some meat exudate in its packaging. This exudate or meat juice contains blood and electrolytes and, apparently, protects *Arcobacter* spp. from damage during storage at low temperatures. High rate of survival of *A. butzleri* in chicken meat juice at low temperatures was demonstrated by researchers from Denmark (Kjeldgaard et al., 2009).

Studies from different parts of the world have been reporting *Arcobacter* spp. as contaminants of chicken meat. For example, a 2011 Indian study revealed *Arcobacter* contamination in a variety of samples that also included chicken (12%) (Patyal et al., 2011). *A. butzleri* was isolated from 26.5% of Ghanian chickens (Paintsil et al., 2021). 45% and 14% of Iranian chicken carcasses were positive for *A. butzleri* and *A. cryaerophilus* isolated in 2014 using enrichment protocol (Khoshbakht et al., 2014). In 2005 a then new species of *Arcobacter-A. cibarius* was isolated from Belgian broilers (Houf et al., 2005). Fernandez et al. found that prevalence of *Arcobacter* spp. on chicken carcasses was much greater than on any other meat parts, which was suggestive of contamination of either the equipment, or the facilities, or both (Fernandez et al., 2015).

3.7. *Arcobacter* spp. in animals

In animals *Arcobacter* species have been implicated in abortive infections and enteritis (Brückner et al., 2020). Not surprisingly, first isolates of *Arcobacter* spp. came from aborted bovine and porcine fetuses (Shah et al., 2011). *A. cryaerophilus* has also been isolated from raw milk sampled from cows with mastitis (Douidah et al., 2012). In non-primate monkeys

Arcobacter spp. cause chronic diarrhea. However, scientific data suggest that representatives of the genus *Arcobacter* could be commensals in some clinically healthy domestic and farm animals, such as cats, dogs, cattle and pigs.

Because of the high body temperature of birds (40-43˚C), it has been suggested that *Arcobacter* spp. may only transiently colonize chickens due to the preference for lower temperatures (26-30˚C) for growth (Fernandez et al., 2015). This is debatable, because not only Atabay et al. reported healthy domestic geese harboring these bacteria, in our laboratory we have grown isolated *A. cryaerophilus* strains microaerophilically both at 37˚C and 42˚C (Atabay et al., 2008).

A wide genotypic variety of *Arcobacter* spp. sometimes exists in animals living in the same household, which may indicate that some genotypes could be more pathogenic than others. Although *A. bultzeri* has been linked with human and animal infections, neither the exact mechanism of pathogenicity, nor the infective dose of this microorganism are known (Uljanovas et al., 2021). On the other hand, the pathogenicity of different *Arcobacter* isolates has been confirmed by studies using human and animal cell culture-based assays: pathogenic *Arcobacter* species, possess *Campylobacter*-like virulence factors responsible for adhesion, invasion and cytotoxic effect, which proceeds with up-regulation of interleukin-8 (Collado and Figueras, 2011).

Taking into consideration the wide variety of sources from which *Arcobacter* spp. have been isolated, it has to be understood that these microorganisms are ubiquitous (Hamill, Neill, and Madden, 2008). Apart from environmental and ground waters, sewage and floodwaters, various food products, such as meats, ready-to-eat salads, and delicacy mollusks, *Arcobacter* spp. have also been isolated from food-processing facilities and food-processing equipment (Di Noto et al., 2018).

Several studies demonstrated association of *A. cryaerophilus* with farm-raised pigs as well as retail pork meats (Kjeldgaard et al., 2009). The most frequent isolate from pork meat was, again, *A. butzleri* followed by *A. cryaerophilus*. A Belgian study conducted in 2004 on healthy pigs at four different farms identified a wide genetic heterogeneity of *Arcobacter*

spp., sometimes several genotypes colonizing one individual. 16 to 85% of pigs were found to be colonized with *Arcobacter* spp. (Van Driessche and Houf, 2007). An Australian study conducted in 2006 confirmed that pig farms may, in fact, be reservoirs for *Arcobacter* spp. By sampling pig effluent ponds and soil treated with effluents, researchers identified that out of total 83 isolates 49% belonged to *A. cryaerophilus* and 35% were those of *A. butzleri* (Chinivasagam et al., 2007).

In 2007, a Belgian study identified the presence of *Arcobacter* on 91% of different parts of pork carcasses following slaughter, most probably due to fecal contamination. Although the predominant species identified in the feces was *A. butzleri*, *A. cryaerophilus* was more prevalent on the carcasses (Van Driessche and Houf, 2007). The study also noted that chilling decreased, but did not eliminate contamination levels. The fact that 10 or more genotypes of *Arcobacter* were isolated from the herds killed on the same day indicated cross-contamination. Notably, samplings of water and worker's boots in this study also yielded *Arcobacter* spp., to reconfirm the importance of water in the transmission of these bacteria (Van Driessche and Houf, 2007). Moreover, water most certainly was the primary source of transmission in this case, therefore future studies should focus on the role of water in the transmission of *Arcobacter* spp. in farms.

Arcobacter spp. have been identified and quantified at 102-104 CFU/g of feces with healthy cattle in a different Belgian study and ranged from 5% to 15% at the three farms examined (Van Driessche et al., 2004). A more recent Italian study focused on sampling milking cows, milk samples, as well as farm surroundings, including other animals, such as pigeons and cats, living on the farm. *A. cryaerophilus* was identified as dominant species (54.2%) in a total of 463 samples., except for raw milk, where *A. butzleri* was the most frequent contaminant (Giacometti et al., 2015).

Due to formation of biofilms, which create protective matrices on various surfaces in the slaughterhouse environment and food-processing equipment, *Arcobacter* spp. can effectively survive disinfection. Biofilm formation was noted at a wide range of temperatures: from 5˚C to 37˚C (Šilha et al., 2021). *A. bultzeri* can survive refrigeration temperatures for at least 3

weeks, as reported by Hilton et al.in 2001 (Hilton et al., 2001). Also, as demonstrated by a Danish study, chicken juice medium enables *Arcobacter* spp. to survive better in the food-processing environment showing less than 1 log reduction after 77 days at 5-10˚C (Hald et al., 2015).

De Smet et al. found that the predominant species of *Arcobacter* isolated from beef carcasses, chilled beef and raw minced beef was *A. butzleri*, although other species, such as *A. cryaerophilus*, were frequently present. Chilling the meat for 24 hours greatly diminished the amount of *Arcobacter spp*. A significant decrease of *Arcobacter spp.* from the initial 37% on pre-chilled bovine carcasses down to 7% on beef was observed post chilling (De Smet et al. 2010). In the same study 9% of minced beef was positive for *Arcobacter spp.* In some countries, such as Belgium, minced meat is eaten raw and this is particularly alarming (De Smet et al. 2010). Although there is probably no absolute single measure of eliminating *Arcobacter spp.* from meat products, good hygiene practices maintained in the production process is probably the most important aspect of meat production.

3.8. *Arcobacter* spp. in domestic animals and pets

Domestic animals may also harbor *Arcobacter* spp.: studies of *Arcobacter* prevalence conducted in Italy (2008) and Belgium (2007) examined cats and dogs as potential carriers of *Arcobacter* spp. While no cats tested positive for *Arcobacter* spp. in the Belgian study, 79% of cats were carriers of either *A. bultzeri* or *A. cryaerophilus* (66% and 29% of the positive population, respectively) in the Italian study (Houf et al.,2007). Only a small percentage (2.6%) of the Belgian dogs carried *Arcobacter*. These findings indicate that pet animals may be able to contribute to the spread of *Arcobacter* species in the domestic habitat and that their recovery may be affected by geographic and seasonal variations. Seasonal variations may, in fact, play an important role in the prevalence of *Arcobacter*. Additionally, as mentioned before, recovery of *Arcobacter* spp. from animals in different geographic regions are greatly affected by nutritional factors and methods of isolation.

3.9. *Arcobacter* spp. in the environment

Increasing evidence suggests that transmission of *Arcobacter* spp. is strongly associated with contamination of environmental and ground waters with human and animal fecal matter (Collado et al., 2008). Microorganisms of the genus *Arcobacter* share the ability to form biofilms with *Campylobacter* spp. Additionally, their aerotolerance and the ability to grow at temperatures below 30°C, better adapt *Arcobacter* spp. to different environmental conditions. A 2004 study that tested survival of *A. butzleri* NCTC 12481 in chlorinated and unchlorinated water revealed that, while chlorinated water resulted in the attenuation of this microorganism after 5 minutes, the bacterium remained culturable for 16 days in untreated water (Moreno et al., 2004). In comparison, *A. tbilisiensis* survived for 10-14 days in river water at refrigerated temperatures (refer to the methods and result sections). These facts explain isolation of *Arcobacter* spp. from environmental and drinking waters suggesting that water plays the key role in their transmission. A 2008 study of the marine environment conducted in Messina, Italy, which isolated *Arcobacter* spp. from surfaces of planktonic copepods also supports the evidence about the special relationship of *Arcobacter spp*. with aquatic environments (Gugliandolo et al., 2008). Waters from river estuaries in Southern Italy also yielded cultures positive for *A. cryaerophilus* from 75% samples tested, although 100% of the samples were positive when tested by either PCR or FISH(Fera et al. 2010).

Sequences of *A. butzleri* genome showed high similarity to *Sulfuromonas denitrificans*, *Wolinella succinogenes* and a deep-sea bacterium *Sulfurovum nitratiruptor*, thus revealing dependence of *Arcobacter* spp. with environments dominated by water (Miller et al., 2007). Environmental waters serve as significant reservoir for *Arcobacter* species. For example, *A. butzleri* and *A. cryaerophilus* were isolated from 55% seawater and freshwater samples in a Spanish study (Collado et al., 2008). Again, the dominant species was *A. butzleri* (94%) followed by *A. cryaerophilus* (30%). Similarly, 30% of bivalve mollusks from the Adriatic sea were carriers of *A. bultzeri* and *A. cryaerophilus* (Leoni et al., 2017). Moreover, in salty marshes another representative of the genus *Arcobacter- A. nitrofigilis-* is associated with plant roots as a nitrogen-fixing bacterium, while a few other species are free-living

environmental bacteria (Ho et al., 2006). Sampling of lake Erie beaches in Ohio, US, revealed significant contamination of waters by *A. cryaerophilus* and its density in the lake significantly correlated with the human-specific fecal marker HuBac by Spearman's correlation analysis (Lee et al., 2012). These facts indicate that contaminated water and food indeed play a very important role in the transmission of *A. cryaerophilus* and may in fact be the main routs of transmission of *Arcobacter* spp., especially when considering that, besides animal feces, *Arcobacter* spp. have been isolated from human fecal samples. For example a German study, which analyzed 4636 fecal samplings from inpatients and outpatients, identified *A. butzleri* as he most frequently isolated microbial species, followed by *A. cryaerophilus* (24.7% and 10.3%, respectively). *A. lanthieri* was isolated to a lesser extent and, while all three microorganisms were found in the outpatient samples, the inpatient samples allowed for the isolation of *A. butzleri* alone (Shah et al., 2011; Brückner et al., 2020). *A. butzleri* also predominated other *Arcobacter* spp. in the stools of Belgian and French patients, as well as in patients with gastrointestinal symptoms in South Africa (Collado et al., 2008).

3.10. Antimicrobial resistance in *Arcobacter* spp. and *Campylobacter* spp.

Macrolides, fluoroquinolones, aminoglycosides and tetracyclines have usually been effective against *Arcobacter* spp., while reduced susceptibility to these antibiotics have also been noted (Uljanovas et al., 2021). This is especially true for fluoroquinolones. Until recently, ciprofloxacin was considered a drug of choice for treating *Campylobacter* infections (Pedonese et al., 2017).

In general, about 40% of *Campylobacter* isolates worldwide are resistant to fluoroquinolones (Kinana et al., 2007). The problem with the drug-resistant foodborne bacteria is that they have a selective advantage in those patients who were already treated with antibiotics for different reasons. These circumstances result in increased transmission of such pathogens (Hashempour-Baltork et al., 2019). In the early 1990s several Asian (China, Thailand, Vietnam) and European countries (Sweden, Spain and the Netherlands) introduced the

veterinary use of quinolones. This coincided with primary resistance to fluoroquinolone therapy in patients in the same countries (Alfredson and Korolik, 2007).

In a 2016 Belgian study the majority of *A. butzleri* isolates (87%) were susceptible to ciprofloxacin, however more than half of *A. cryaerophilus* strains (51%) were resistant to this drug (MIC >32 mg/L) (Van Den Abeele et al., 2016). All ciprofloxacin-resistant strains carried the same mutation in the *gyrA* gene. Mutation in the QRDR region (gyrA) gene was also found in some of the *Arcobacter* isolates identified in our study.

Interesting results were obtained by a research group of the University of Palermo, Italy, who isolated *Arcobacter* spp. from environmental waters and tested their antibiotic susceptibility.The majority of isolates (96%) belonged to *A. butzleri*, while *A. cryaerophilus* isolates comprised the remaining 4%. All strains were resistant to tetracycline, nalidixic acid and β-lactam antibiotics, such as ampicillin, cefalotin and cefotaxime. A few *A. butzleri* strains isolated from seawater, seaweed, and river water were resistant to both erythromycin and ciprofloxacin. One *A. butzleri* isolate was identified as resistant to ciprofloxacin alone. At the same time, all *A. cryaerophilus* isolates were resistant to all tested antibiotics, except for gentamicin. In 2020 Czech scientists identified that the 60 strains of *Arcobacter* spp. they had isolated from different sources, including water, were highly susceptible to gentamicin (98.3%), ciprofloxacin (95.0%), and erythromycin (100.0%). The majority of the *Arcobacter* isolates were susceptible to ciprofloxacin in a 2020 Estonian study as well (Uljanovas et al., 2021). On the other hand, high levels of resistance were noted to clindamycin and tetracycline while combined resistance to both clindamycin and tetracycline was observed in 38.3% of the isolates (Šilha et al., 2021). A Tunisian study of 2020 identified that among chicken isolates of *Arcobacter*, where *A. butzleri* predominated, all 24 *A. butzleri* strains were significantly resistant to erythromycin (P = 0.0015), ampicillin (P = 0.001), and ciprofloxacin (P = 0.05). All 4 *A. cryaerophilus* isolates were susceptible to ampicillin, amoxicillin-clavulanic acid and gentamicin. 83% of all *Arcobacter* spp. were identified as MDR, which is a serious public health concern (Jribi et al., 2020).

3.11. Mechanisms of antimicrobial resistance in *Campylobacter* spp.

Macrolides or fluoroquinolones have been the antibiotics of choice for the treatment of C*ampylobacter* infections, however resistance to both groups of antimicrobials has been on the rise (Aleksić et al., 2021). Even to this day, both fluoroquinolones and macrolides are frequently used to treat campylobacteriosis (Abd El-Tawab et al., 2019).

The World Health Organization (WHO) recognizes antibiotic resistance in medicine and agriculture as a major public health concern worldwide. With the prospect of therapeutic failures of life-saving treatments antibiotic resistance has a potential to become a global challenge to human and veterinary medicines (Koga et al., 2017). Increased virulence resulting in longer duration of illness has been demonstrated in studies of infections caused by pathogenic bacteria that are drug-resistant (Michaelis and Grohmann, 2023). Resistant strains of *Campylobacter* spp. can prolong infections and make treatment ineffective, especially in the immunocompromised patients, where the incidence of campylobacteriosis is higher (Bungay et al, 2005).

There are two reasons why *Campylobacter* spp. in general, and their resistance to antimicrobials in particular, should be of concern: first, *C. jejuni* and *C. coli* can evolve rapidly and adapt fast due to their large populations, even though de novo mutation rates in this microorganisms are rare. Second, horizontal gene transfer helps these pathogens acquire a large number of polymorphisms simultaneously. Such resistant lineages of both *C. jejuni* and *C. coli* have an advantage over their competitors and can expand locally very fast, as both species easily adapt to multiple hosts (Sheppard and Maiden, 2015).

One of the circumstances contributing to drug resistance of bacteria is that the genes encoding for antimicrobial resistance are frequently located on mobile genetic elements: plasmids, transposons, and integrons, which can be horizontally transferred to other bacteria. However, in bacteria antimicrobial resistance is mostly plasmid-mediated (Marasini et al., 2018). *Campylobacter* species are characterized with genetic mechanisms conducive to natural transformation and conjugation: once acquired, antimicrobial resistance genes are readily transferred to new strains. Such heterologous genetic exchange with Gram-positive

cocci, for example, led to the incorporation of *tet(O)* and *aphA-3* genes into the *Campylobacter* genome (Wieczorek and Osek, 2013).

Out of the two mechanisms of antimicrobial resistance (AMR)-intrinsic and acquired-the latter, which results in a point mutation of the *gyrA* gene targeted by the drug, is involved in the resistance to fluoroquinolones in *Campylobacter* spp. (Wieczorek and Osek, 2013). Within the quinolone resistance-determining region (QRDR) of *gyrA*, the most frequent mutation associated with high levels of resistance to fluoroquinolones is a C to T transition in codon 86 resulting in Thr-86-Ile substitution (Kinana et al., 2007).

Due to their strong association with mobile genetic elements, such as plasmids and transposons, integrons also play a major role in horizontal gene transfer (HGT), particularly in the exchange of drug resistance genes. Integrons are thought to be involved in multi-drug resistance due to their ability to localize and express MDR genes. Since their first discovery in the 1980s, integrons have been identified in numerous Gram-negative and Gram-positive bacteria (Piccirillo et al., 2013). The HGT mechanisms in *C. jejuni* and *C. coli* are not yet completely clear, however class 1 integrons have already been identified in both human and animal isolates of both species. Despite the evidence of class 1 integrons in *Campylobacter* spp. their presence have been demonstrated only in limited number of strains. An Italian study that analyzed sequences of 362 strains of *C. jejuni* and *C. coli* has not identified any class 1 or 2 integrons in their strains, which were highly resistant to fluoroquinolones, ampicillin, cephalosporins and tetracycline. Therefore, integron-mediated mechanism of AMR may be quite rare in *Campylobacter* spp. (Piccirillo et al., 2013).

Besides mutations in the gyrA-encoding subunit of the DNA gyrase, other factors, such as nodulation cell division superfamily (RND) efflux pump-contribute to both intrinsic and acquired resistance to fluoroquinolone in *Campylobacter* spp. (Nikaido and Takatsuka, 2009).

Today emerging *Campylobacter* strains resistant to various antimicrobials is a major public health concern in a number of countries due to the use of antibiotics in animal feed (Wagley et al., 2014). In their 2007 publication, Alfredson and Korolik suggest that the use of

ciprofloxacin for the treatment of *Campylobacter* infections is no longer advisable due to high rates of resistance resulting from indiscriminate use of antibiotics in humans and animals (Alfredson and Korolik, 2007). To this date, however, the use of antimicrobials in animal and poultry farms still continues in many countries to prevent and control infections and even to enhance growth of food animals. As a result, *C. jejuni* and *C. coli* are already resistant to penicillins, the 1st and 2nd generation cephalosporins, trimethoprim, sulfamethoxazole, rifampicin and vancomycin (Wieczorek, Bocian, and Osek, 2020). Even though the sub-therapeutic use of antibiotics has been banned in European countries, the United States still use antimicrobials for enhancing growth of food animals. Phasing out of antimicrobials for this purpose was "recommended" by the current guidance to the industry, however it is not legally binding (FDA, 2013). Thus, the main mechanism of antimicrobial resistance is still due to their use as growth promoters in food animals (Alfredson and Korolik, 2007). Studies have established a direct connection between resistance profiles found in *Campylobacter* spp. isolated from broilers and the antimicrobials used in the breeders (Tang et al., 2020). Other studies also have linked the use of antimicrobial agents, in particular fluoroquinolones, in the agricultural industry and veterinary medicine, to the emergence and spread of resistance among *Campylobacter* strains (Iovine, 2013) Techniques, such as Pulse Field Gel Electrophoresis and Random Amplified Polymorphic DNA method were used to match resistance profiles of the broiler isolates to those of the breeders' revealing vertical transfer of resistance (Han et al., 2016).

Resistance to tetracycline is frequently reported in *Campylobacter* spp. and, in most cases, it is caused by the presence of tet(O) gene. Indeed, due to its low cost tetracycline is the most widely used antibiotic in avian production. With years, however, its effectiveness has been decreasing, as its microbial spectrum has been narrowing (Wieczorek and Osek, 2013). Among the isolates of *Campylobacter* spp.-51% and 96% of *C. jejuni* and *C. coli*, respectively-were found to be resistant to tetracyclin in this study. The *tet(O)* gene seems to have global presence and has been detected in many parts of the world. For example, a study

from Ireland identified that 100% of the chicken isolates of thermophilic *Campylobacter* spp. were harboring *tet(O)* (Lynch et al., 2020).

Emergence of multidrug resistant *Campylobacter* spp. is also worrisome. In a study conducted in Ireland more than 24% of 290 *C. jejuni* isolates were resistant to more than two drugs (Madden et al., 2011). Resistance was detected to ceftifur (58%), ampicillin (25%), nalidixic acid (17%), streptomycin (7.9%) and chloramphenicol (8.3%). At the same time, 80% of human *C. jejuni* isolates were found to be resistant to the cephalosporin ceftifur (Madden et al., 2011). In a similar study conducted in Poland, 91% of *C. jejuni* isolates were resistant to ciprofloxacin (Wieczorek and Osek, 2013). An Indian study observed that the highest rate of resistance among the *C. jejuni* isolates from chicken meat was to nalidixic acid (81.25%) and ciprofloxacin (63.46%). The isolates were also resistant to tetracyclin (41.34%), amoxicillin (31.25%) and colistin (37.01%). Resistance to neomycin, ampicillin, chloramphenicol, erythromycin and gentamicin was found to be 11.05%, 15.86% 6.73%, 5.76% and 3.84%, respectively (Sathiamoorthi et al., 2016). These data show that antimicrobial resistance among *Campylobacter* spp. is growing, perhaps partly due to ineffective regulation of antimicrobial use in humans and animals in developing countries. Indiscriminate use of antimicrobials was cited as the cause of resistance of the *C. jejuni* isolates to chloramphenicol among the isolates obtained from the US troops in Thailand (Bungay et al., 2005).

Prevalence of *Campylobacter spp*. resistant to various antimicrobial drugs varies from country to country. For example, an Italian research group that investigated prevalence and genotypic diversity of *C. jejuni* and *C. coli* in fresh retail chicken in Tuscany identified that prevalence of *Campylobacter* spp. in the meats exceeded 60% with roughly the same distribution of *C. Jejuni* and *C. coli* (42% and 58%, respectively) and high resistance to tetracycline, ciprofloxacin and nalidixic acid (79.1%, 72.1% and 65.1%, respectively). 14% of *C. jejuni* strains in this study were found to be resistant to both erythromycin and ciprofloxacin. In a 2011 Polish study, ten out of the 143 *Campylobacter* strains (7.0%) turned out to be resistant to 3 unrelated antimicrobials. High rates of resistance to ciprofloxacin

were observed among the isolates of both species (63% for *C. jejuni* and 72% for *C. coli*) and tetracycline (42% and 43%, respectively) (Andrzejewska et al., 2011). High incidence of fluoroquinolone resistance was also seen in both species (100% and 98.9% for *C. jejuni* and *C. coli*, respectively) in a study conducted in Thailand. Tetracyclin resistance was high as well (98% and 56%). Additionally multi-drug resistance was observed in most isolates (Thomrongsuwannakij et al., 2017).

The levels of resistance to quinolones among *Campylobacter* spp. may vary geographically. For example, a study conducted by the Prince Leopold Institute of Tropical Medicine in 2007 identified that the resistance rates to ciprofloxacin among the samples taken between 1994 and 2006 increased significantly among travelers returning from Asia, Latin America and Africa. The highest rates of resistance were identified in travelers from Asia (71%), followed by Latin America (61%) and Africa (31%) (Vlieghe et al., 2008).

Antibiotic resistance finds its way to wild bird populations. For example, a 2015 Polish study sampled 398 of white storks chicks, out of which 5.3% and 2.3% of samples were positive for *C. jejuni* and *C. coli* with 52.4% and 44.4% resistance, respectively, to ciprofloxacine. Additionally, 19% of *C. jejuni* and 77.8% of *C. coli* were resistant to tetracycline (Szczepańska et al., 2015).

3.12. Control and risk management of *Campylobacter* spp. in poultry

Due to ubiquitous presence of *Campylobacter* spp. *and Arcobacter* spp. in the environment, strict farm biosecurity measures are perhaps the most significant factor in decreasing their prevalence in farm-raised poultry, followed by informing consumers about the risks associated with these infections (Skarp et al., 2016).

Scientific data suggest that *Campylobacter* colonization in hen occurs without presenting any clinical signs and as early as 14-21 days. Infection starts with low percentage and increases to a high contamination level by the time chicks have grown (Guyard-Nicodème et al., 2016; Tang et al., 2020). Presumably, maternal IgG antibodies that are transferred from the mother's serum to the egg yolk protect the chicks from infections during the first weeks before their own immune systems kick in. From two weeks of life onward, anti-

Campylobacter antibody concentrations drops significantly in the chicks' blood coinciding with susceptibility of the chickens to *Campylobacter* colonization. While vertical (from parents to chicks) transmission events of *Campylobacter* are rare, horizontal transmissions are widespread: flocks in farms with intensive production often vary between 10,000–30,000 birds, which facilitates rapid spread of *Campylobacter* spp. horizontally. Reports regarding vertical transmission of *Campylobacter* infections are contradictory: the fact that *Campylobacter* spp. have been found in 2-day old chicks makes vertical transmission plausible, however a study that tracked 60 thousand chicks hatched from the eggs of colonized mothers did not identify any evidence of such transmission (Silva et al., 2011). *Campylobacter* infections within a flock take place amazingly fast: a single chicken that has been infected can further infect almost 100% of the flock in one week (Vandeputte et al., 2019). Once a *Campylobacter* infection has been established after an instance of a rapid horizontal transmission, eradication of the infection becomes impossible (Silva et al., 2011).

The slaughter process is one of the important stages in primary production. Cross-contamination of chicken carcasses with *Campylobacter* spp. usually occurs during this stage (Tang et al., 2020). After chickens are killed, the broiler carcass will inevitably get contaminated with the intestinal content. Equipment used in this process will further introduce *Campylobacter* spp. into chicken meat (Tang et al., 2020). One of the ways to decrease the spread of *Campylobacter* between the infected and uninfected individuals, is to perform testing and separate chickens into *Campylobacter*-positive and *Campylobacter*-negative flocks. The contamination level may be determined by testing a flock twice: at 4 weeks and a day or two before the slaughter. Since there is no adequate convenient method of *Campylobacter* quantification in broiler chickens antemortem, this testing method would allow for a fast and simple determination of presence of *Campylobacter* spp. in a particular flock and take appropriate measures to control this pathogen (Tang et al., 2020). Instead of taking cloacal swabs, a German study suggested using qPCR sampling of boot socks in order to detect *Campylobacter* spp., which is a usual practice for *Salmonella* screening in chicken industry in Europe. Additionally, testing prior to killing allows the producer to determine

highly positive flocks, which can be frozen and, thus, neutralized. This model was successfully used in Iceland leading to a significant drop in *campylobacteriosis* cases in the country (Haas, Overesch, and Kuhnert, 2017). Denmark also successfully used a similar model, which resulted in the production of certified *Campylobacter*-free chicken meat in the country (Silva et al., 2011).

To prevent transmission of *Campylobacter* spp. to consumers, poultry farms need to introduce strict hygienic measures. The probability of contamination of meat during processing is very high: from the carcasses of the colonized birds *Campylobacter* spp. will inevitably end up on the processing equipment, thus contaminating *Campylobacter*-free carcasses. To prevent this, thorough decontamination of equipment is mandatory in processing facilities. Good Hygienic Practices introduced along the processing line and at the farms in general have been effective in reducing campylobacter (Silva et al., 2011). Some processing plants revert to treating chicken meat with lactic and/or acetic acids achieving some reduction of *Campylobacter* spp. It is recommended not to wash a store-bought chicken when cooking at home, while in catering kitchens washing the processing areas and utensils with hot water and hypochlorite has been shown as effective (Silva et al., 2011). In farms, a variety of hygienic measures and feeding practices should be taken to effectively decrease colonization of birds with *Campylobacter* spp. For example, installing hygienic barriers and restricting farm access to limited personnel, monitoring drinking water and feed for farm chickens, eliminating animal protein in chicken feed etc. All these measures have been shown to be effective for preventing *Campylobacter* colonization of farm poultry, although without its complete elimination (Silva et al., 2011).

3.13. Control of *Campylobacter* spp. in primary production

The fact that *Campylobacter* spp. are found in the environment and, as symbionts, in both domestic and wild animals, makes it especially challenging to control these bacteria in farm-raised chickens. Antimicrobials use is not advisable, partly because of the inherent resistance of *Campylobacter* spp. to some antimicrobial drugs and partly because of the acquired resistance to others. Another problem is that residual antimicrobials, for example

sulfonamides, aminoglycosides and macrolides, have been found in meats and fish. Liquid chromatography and mass spectrometry can both be applied to detect residual antimicrobials of veterinary use (Ikai et al., 1991). The veterinary antimicrobial drugs widely used to treat infections in animals can be detected in meat products and European Union has introduced minimum residue limits in order to protect consumers (Berrada et al., 2010). This leaves the manufacturers and veterinarians with limited choices, one of these being to combine available measures in order to decrease *Campylobacter* infections in farm-raised chickens. In conjunction with improving the sanitation conditions and introducing strict biocontrol measures at farm facilities, either phage therapy or probiotic cocktails, or the combination of the two, may be used.

3.14. Use of LAB supplements in biocontrol of *Campylobacter* spp.

To decrease the number of *Campylobacter* spp. circulating within poultry farms without the use of antimicrobials, prebiotics (fructo-and galacto-oligosacharides) and probiotics (*bifidobacteria* and *lactobacilli*) may be added to chicken feed separately, or as a combination formula. In vivo experiments using such products have already been conducted with certain success (Guyard-Nicodème et al., 2016). For example, PoultryStar® reduced *Campylobacter* by 1.88 log10 CFU/g in chickens in vivo (Guyard-Nicodème et al., 2016). One of the mechanisms of action of probiotic bacteria in fighting various pathogens is their ability to generate antimicrobial compounds. These compounds may be divided into two major categories: the first category consists of organic acids of low molecular mass, usually below 1,000 Da, while the second group is represented by larger molecules of peptide or protein nature, known as bacteriocins (Neal-McKinney et al., 2012),(Yang et al., 2014).

While reviewing the literature on the probiotic bacteria inhibiting pathogenic bacterial species in vitro, it became evident that most authors believed that the antimicrobial activity of probiotics resulted from the production of organic acids by the latter, due to oligosaccharide fermentation. In recent years, however, another category of antimicrobial compounds-bacteriocins-have been gaining prominence in research. It has been demonstrated that probiotics successfully inhibited *Salmonella*, *Shigella*, *Listeria*,

Helicobacter, *Escherichia* and other species in a manner that could not be attributed exclusively to organic acids and the resulting low pH of the co-incubation media (Ahmad and Aqil, 2008).

When bacteriocins were first identified, the predominant opinion was that they were effective only against the species closely related to the producer bacteria. For example, a 1984 publication that identified and described Lactacin B-a low molecular weight bacteriocin of about 6,500 Da produced by *Lactobacillus acidophilus*-demonstrated its activity against the representatives of the same family-*L. leichmannii, L. bulgaricus, L. helveticus,* and *L. lactis.* Lactacin B was sensitive to proteinase K, indicating its protein nature and insensitive to chlorophorm, which showed that it did not contain any lipid (Barefoot and Klaenhammer, 1984).

Today there is growing evidence that bacteriocins can affect a wider spectrum of organisms and even act as signaling molecules (Klijn, Mercenier, and Arigoni, 2005) An excellent example is Plantaricin MG-a 2180 Da bacteriocin produced by *Lactobacillus plantarum* KLDS1.0391 isolated from traditional Chinese fermented cream. Plantaricin MG demonstrated a broad inhibitory activity against both Gram-positive and Gram-negative bacteria, including *Listeria monocytogenes* and *Salmonella typhimurium*. Another LAB, *Bifidobacterium bifidum,* produces a broad-spectrum bacteriocin Bifidocin B and the genome sequence of *B. longum* DJO10A revealed two coding sequences (CDS) that potentially produce lantibiotics-small bacteriocins that consist of lanthionine and beta-methyl-lanthionine (Klijn, Mercenier, and Arigoni, 2005). Screening for probiotic properties requires characterization of both the probiotic strain and the pathogenic strain, followed by selection of the most effective probiotic strain that can be used for therapeutic purposes (Varankovich, Nickerson, and Korber, 2015). A French research group did just that having isolated 45 LAB isolates from chicken feces. Characterization of these isolates using molecular methods and API panels resulted in the identification of *L. reuteri* active against *C. jejuni* NCTC 11168 (Nazef et al., 2008).

Many studies have demonstrated some level of Inhibition of *Campylobacter* species by LAB in vitro. Most attributed such inhibition to the ability of *Bifidobacteria* and *Lactobacilli* to produce organic acids thus decreasing the pH of the surrounding media (Meremäe et al., 2010). These are mostly lactic and acetic acids that accumulate in in-vitro systems thus making the system pH drop below what *Campylobacter* can normally tolerate. Quite similar results were obtained by Bratz et al. in a study that used a well-diffusion agar assay to assess the ability of probiotics to inhibit *C. jejuni* in vitro. The authors concluded that the observed inhibition was due to the low pH of the cell-free supernatant: whenever the pH was neutralized, inhibition was no longer observed (Bratz et al., 2014). However, the relationship between the pH of the cell free medium and the observed inhibition does not always appear to be straightforward. For example, in the instance of co-incubation of *Campylobacter* spp. *with L. reuteri* (pH 4.3), *C. coli* and one of the two *C. jejuni* isolates used in the study were not inhibited, while the cell free preparations of other lactobacilli inhibited *Campylobacter* spp. at the pH close to 4.3 (pH 4.0 and pH 4.1, respectively) (Bratz et al., 2014). In a study conducted in 2002, Fooks and Gibson observed that cell free preparations of *Lactobacillus plantarum* and *Bifidobacterium bifidum* supplied with oligofructose (FOS) and xylo-oligosaccharide (XOS), or the combination of the two, effectively inhibited enteric pathogens, such as *C. jejuni* ATCC 11351 and *E. coli* NCIMB 9517 (Fooks and Gibson, 2002). Interestingly, in disc diffusion assays the cell free media were more effective compared to the cell fraction. In some cases the pH was lowered as soon as 3 hours, however, inhibition did not take effect until after 9–24 h. Some studies have put forward a hypothesis that the inhibitory activity observed during co-incubation of probiotics with *Campylobacter* spp. could, besides low pH, result from the synthesis of antibacterial peptides by lactic acid bacteria (Bratz et al., 2014). Meremäe et al. noted in a study conducted in 2010 an increase in the concentrations of organic acids in co-incubation media (Meremäe et al., 2010). The inhibitory effect was observed against all *C. jejuni* strains tested and while the authors stated that the inhibition was due to acidic pH, they noted that the inhibition could also be resulting from unknown antimicrobial factors (Meremäe et al., 2010).

The intestinal pH in living birds is approximately 5.5. When conducting in vitro experiments, one must take into consideration that in vivo systems are infinitely more complex as in living systems many different factors determine gene regulation (Ravindran 2013). As demonstrated in a study conducted by Kral et al. in the experimental and control groups of chickens, a 30 min incubation of *C. jejuni* NCTC 11168 at pH 3.75 did not affect bacterial viability (Král M. et. al 2012). On the contrary, there have been reports that short-term exposure to acid causes up-regulated expression of certain invasion factors, e.g. FlaA, in *Campylobacter* spp. by driving these pathogens into survival mode (Le et al. 2012). Thus, exposure of *Campylobacter spp.* to low pH results in its increased invasiveness. During fecal-oral route of infection *Campylobacter* spp. must pass through the environment of extremely low pH (pH 1.5–2) of the stomach and still be able to infect the host. Scientists think hat the acidic environment of the stomach primes these bacteria for increased invasiveness (Le et al., 2012).

Some probiotics affect the ability of *Campylobacter* spp. to invade intestinal epithelial cells in vitro. For example, invasion of T84 human colon cancer epithelial cells and human embryonic intestine 407 cells by *C. jejuni* NCTC 11168 and *C. jejuni* ATCC 81–176 was inhibited by 41% and 35%, respectively, after pretreatment of these cells with *L. helveticus* for 1 hour (Wine et al., 2009). In contrast, heat-killed *L. helveticus* reduced the inhibition by 24 ± 8% and 27 ± 9%, respectively, indicating that competitive exclusion was not the only mechanism taking place, although *L. helveticus* did successfully adhere to both intestinal cell lines (Wine et al., 2009). Another finding of the same study suggested the strain-specific nature of such inhibition. For example, *L. rhamnosus* did not inhibit *C. jejuni* NCTC 11168. At the same time, this probiotic reduced the invasiveness of *C. jejuni* ATCC 81-176 by 37%, compared to the unprotected control sample. This suggests that the protective effect of probiotics depended on both the probiotic and the pathogen strains (Wine et al, 2009). Several research groups demonstrated in clinical studies that some lactobacilli can modulate the immune response of the host organism. For example, *L. johnsonii* La1 enhanced the phagocytic activity towards *E. coli* in healthy volunteers that

ingested fermented milk. La1 also increased secretory IgA levels against *Salmonella typhi* Ty21a-an oral vaccine designed to mimic an enteric infection (Dicks and Botes, 2010).

More research is needed to ascertain the mechanisms of inhibition of enteric pathogens, such as *Campylobacter* spp*.,* by LAB. Although the pH-dependent mechanism is an obvious and easier explanation of such inhibition, a more specific, bacteriocin-mediated mechanism may also be taking place. LAB may directly affect *Campylobacter* spp., for example by producing peptides or proteins similar to the mechanism identified by Fujiwara et al. against *E. coli* strain Pb176 (Fujiwara et al., 1999).

4 Materials and Equipment

4.1. Equipment

4.2. Cell Culture, Reagents and Supplements

D (DMEM supplemented with 2% of fetal calf serum	Karlsruhe, Germany
Trypsin 0.05%	Karlsruhe, Germany
Gibco Phosphate buffer solution, sterile	Karlsruhe, Germany
CACO-2 cells-Human Colon carcinoma cell line	Institute of Medical Microbiology cell bank
Roche Sigma Aldrich WST1 Cell Proliferation Reagent	Taufkirchen, Germany
Gibco Penicillin Streptomycin Supplement	Karlsruhe, Germany

4.3. Media and Reagents for Microbiology

BioLife Campylobacter Blood Free Medium Base	Milan, Italy
Oxoid Columbia blood agar	Wesel, Germany

Deltalab Gram's Staining Kit	Barcelona, Spain
Deltalab Carbol fuchsin stain	Barcelona, Spain
GMP Cefoperazone 1 g	Tbilisi, Georgia
BioLife Bolton broth supplemented with laked horse blood	Milan, Italy
BioLife Mueller Hinton Broth	Milan, Italy
BioLife M17 broth	Milan, Italy
C-broth (MH/M17 (75%/25% W/W))	Made in-house
BioLife Mann Rogosa Sharpe (MRS) Agar	Milan, Italy
Liophilchem Latex Agglutination Test for *C. jejuni*	Roseto degli Arbuzzi, Italy
BioLife MRS broth	Milan, Italy

5 Methods

5.1. Sample collection and Isolation of *Arcobacter spp.* and *Campylobacter* spp.

The majority of samples (n=200) were collected over the two-year period from fall 2018 to fall 2020. Some samples (n=46) were added later, during the summer 2021. Whole chicken carcasses, chicken breasts, thighs and livers were purchased in supermarkets or direct sales points of four different producers around Tbilisi.

The purchased whole chicken carcasses were washed with sterile PBS. The wash was collected into sterile 50 mL tubes, after which the tubes were centrifuged at 3200 RPM for 10 min. Pellets were resuspended in 2 mL sterile peptone water and incubated at 37˚C for 2 h. After incubation 100 µL of each sample was plated using the four quadrant method on 4 different CCDA agar plates containing Campy supplement (cefoperazone and amphotericin B). The plates were then incubated at 37˚C for 48 hours. After incubation, colonies resembling those of *Campylobacter* were stained using Gram Staining Kit. Positively

identified colonies were inoculated on a new CCDA agar several times until monoculture was obtained. Other samples, were processed in a similar manner.

In other instances, chicken livers were minced in sterile peptone water in a 50 mL tubes, incubated for 2 hours, after which the solids were separated and 100 μL of the liquid was plated on four different CCDA plates using the four quadrant method.

Chicken breasts were sampled using sterile cotton swabs, which were pre-incubated in Bolton broth for 2 hours prior to inoculation of CCDA agar plates. In parallel, 100 μL of chicken juice samples found in the packages were inoculated on a CCDA plate directly without pre-incubation.

5.2. Conventional light microscopy

CCDA agar was used for isolating both *Arcobacter* spp. and *Campylobacter* spp. For this reason, agar plates were prepared once or twice a week, according to the manufacturer's instructions, to avoid spoilage. A resuspended Campy antibiotic supplement was added to the agar and mixed in after cooling to 50°C. Plates were then poured and stored at 4°C until needed. We compared incubation at 37°C with incubation at 42°C and discovered that at 37°C we had a better rate of isolation.

Microscopy of the cultures were at first performed using Gram's staining kit. However, later we relied solely on fast staining of suspected colonies with carbol fuchsin and observation of curved, S-shaped and/or comma-shaped bacteria. Speciation of *C. coli* and *C. jejuni* can not be reliably done with the use of conventional methods, such as microscopy and colony characteristics. In some cases, colonies of *C. jejuni* and *C. coli* could be differentiated by color and shape. However, this information alone is not reliable. Liophilchem's *C. Jejuni* latex agglutination test also gave variable performance, often producing a positive result with both *C. jejuni* and *C. coli* and thus was deemed as unreliable.

In many instances both *C. jejuni* and *C. coli* could be isolated from the same chicken carcass. *C. jejuni* colonies differed from those of *C. coli* by the colony shape and color: off white and spreading droplet-like colonies of *C. jejuni* versus smaller, discreet, gray-brownish bead-like

colonies of *C. coli.* However, we noticed that after repeated inoculation colonies could change morphology on CCDA agar and this need to be observed and investigated further.

5.3. Culturing *Lactobacilli*

LAB strains were cultured on MRS agar under microaerobic conditions. LAB Colonies generally appeared after 48 hours of incubation. Gram's staining kit (Deltalab, Barcelona, Spain) was used to identify gram positive rods of various sizes. 37 various LAB strains isolated from Matsoni-traditional Georgian yogurt obtained from different regions of the country were kindly provided by Dr. Nina Chanishvili of the Eliava Institute of Microbiology, Bacteriophages and Virology. Additionally, many strains were isolated in our laboratory from different fermented products, such as pickled cabbages, cucumbers, sour milk and buffalo yogurt. 14 strains of *Lactobacillus plantarum* isolated from local sustainably produced apples were provided by Dr. Nino Gagelidze.

All cultures were frozen either in MRS (*Lactobacilli*) or MH (*Campylobacter* spp. and *Arcobacter* spp.) broth containing 15% glycerol: in a clean biosafety cabinet a few bacterial colonies were transferred into the culture broth in a sterile 1.5 mL microfuge tube directly from a monoculture plate. Bacteria were then resuspended with a 200 µL pipette set at 100 µL and using a sterile filtered pipette tip. The tubes then were stored at -70˚C freezer until needed.

5.4. Identification of the isolates by MALDI-TOF MS

All Arcobacter, Campylobacter, and Lactobacilli isolates were subject to Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry performed on a Vitek-MS mass spectrometer, (Biomérieux, Nürtingen, Germany) at the Institute for Medical Microbiology and Hospital Hygiene of the University Hospital Magdeburg.

Briefly, bacterial cultures were plated on Columbia sheep blood agar (Oxoid, Wesel, Germany) and incubated under microaerophilic conditions. After 48 hours, a single bacterial colony from a monoculture was used for identification. For this purpose a colony was touched very lightly with the tip of a sterile toothpick and spread upon predefined spot on a barcoded slide with 48 spots, which were first pre-treated with 5 µL of the α-Cyano-4-

hydroxycinnamic acid (HCCP)-matrix solution. After all samples were transferred on the slide, it was run on the Mass-Spectrometer.

5.5. Co-culture of *L. fermentum* with *Campylobacter* spp.

C-broth was used for co-culture of *Campylobacter* and *Arcobacter* isolates with *L. fermentum*. This medium was useful in maintaining the pH of the co-incubation medium at neutral, while allowing for successful growth of both *Campylobacter* and *Lactobacillus* species. Briefly, colonies of Campylobacter and *L. fermentum* were resuspended, separately, in 1 mL of the C-broth in a sterile microfuge tube. The OD_{600} of the suspensions were measured and adjusted to OD 600 0.1 and 1, respectively. 100 µL of the OD_{600}=0.1 of *C. jejuni* and 50 µL of the OD_{600}=1 of *L. fermentum* were combined in total of 1 mL of the sterile C-broth and incubated at 37°C overnight. After incubation the contents of the microfuge tube was mixed several times and 10 µL of the broth was plated on CCDA in triplicate. The plates were incubated at 37°C for 48 hours under microaerobic conditions, after which growth of the spotted cultures was observed.

5.6. Antibiotic Susceptibility Testing of the *Campylobacter* Isolates

Kirby Bauer disk diffusion method was used to determine antibiotic susceptibility. Testing was performed on all confirmed *Campylobacter* and *Arcobacter* isolates and interpreted according to the guidelines provided by 2022 European Committee on Antimicrobial Susceptibility (EUCAST V13.0).

Antibiotic disks (Oxoid, Wesel, Germany) were placed on Columbia Blood Agar plates inoculated with 0.5 McFarland standard of each respective bacterial strain monoculture. The susceptibility plates were incubated for 48 h at 37°C under microaerophilic conditions, after which the inhibition zones were measured. The zone diameters were interpreted as susceptible (S), or resistant (R) after the EUCAST guidelines (Table 2) (Paintsil et al. 2021).

Because antibiotic susceptibility data is still scarce for *Arcobacter* spp. for the antibiotics tested that did not have EUCAST clinical breakpoint for *Arcobacter* spp., epidemiological cut-off values (Ecoff) established using the frequency distribution of inhibition zone

diameters were used (Table 1). For example, cutoff values for tetracycline, ciprofloxacin and erythromycin were taken from Zautner et. al (Zautner et al. 2023). Developing epidemiological cut-off has been described previously by Bénéjat et al., 2018 (Bénéjat et al. 2018).

Isolates resistant to at least one antimicrobial from each of the following antimicrobial groups- tetracyclines, macrolides, and quinolones-were considered multidrug resistant (MDR), which is defined as resistance to three or more antimicrobials of any substance group.

Table 1: Antimicrobials used in susceptibility testing of *Arcobacter* spp.

Group	Antibiotic	Cutoff
Penicillins	Penicillin G	> 15 mm
	Ampicillin	> 13 mm
Aminoglycosides	Streptomycin	> 11 mm
	Kanamycin	> 13 mm
	Gentamicin	> 19 mm
Macrolides	Chloramphenicol	> 18 mm
	Erythromycin	> 9 mm
Fluoroquinolones	Ciprofloxacin	>13 mm
Tetracyclines	Tetracycline	> 11 mm

Table 2. Breakpoints for determination of antibiotic resistance of *Campylobacter isolates -C. coli and C. jejuni*

Antibiotic (disk concentration)	Zone Diameter (mm)		
	S≥		R<
Tetracycline (30µg)	30		30
Ciprofloxacin (5 µg)	50		26
Erythromycin (15 µg) *C. coli*	20		20
Erythromycin (15 µg) *C. jejuni*	24		24
Ampicillin (10 µg)	13*		7*
Chloramphenicol (30 µg)	18*		18*
Kanamycin (30 µg)	15*		7*
Streptomycin (25 µg)	22*		13*

5.7. Cytotoxicity effect of *C. jejuni*, *C. coli* and the *Arcobacter* isolates on CaCo-2 Cells

To evaluate cytotoxicity of the *C. coli*, *C. jejuni* and *A. cryaerophilus* isolates WSTI cell proliferation reagent (Sigma Aldrich, Taufkirchen, Germany), CaCo-2 cells, all *Arcobacter* isolates and some of the *Campylobacter* spp. were used. The WST-1 assay is based on a simple method, while being an accurate and ready-to-use testing system that enables researchers to measure mammalian cell proliferation, cell viability and cytotoxicity. Specifically, the WST-1 assay protocol is based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases. This means that the larger the number of viable cells, the higher the activity of the mitochondrial dehydrogenases will be, and the greater the amount of formazan dye is formed corresponding to greater OD.

Prior to setting up the experiments, we determined the optimal number of cells to be between 5,000-10,000/well. Additionally, incubation times of 24 and 48 hours were tested

based on the knowledge that *Campylobacter* spp. are slow growers, their doubling time being 48 hours. However, the assay did not show any significant difference between 24 and 48 hours of incubation periods.

5.8. Determination of cytotoxic effect of *Campylobacter* spp. and *Arcobacter* spp. on Caco-2 cells.

CaCo-2s were maintained in DMEM-based cell culture supplemented with 2% FBS. Six locally isolated strains-*C. coli* (n=3), *C. jejuni* (n=2) and *Arcobacter* (n=1)-were used in a cytotoxicity experiment. Prior to the day of experiment, each bacterial strain was re-inoculated on Columbia Blood Agar supplemented with sheep blood (Oxoid, Wessel, Germany). After 24 hours, a medium-sized colony was resuspended from each plate into 1 mL of DMEM in a sterile microfuge tube and then diluted to OD_{600} 0.01. CaCo-2 cells grown to 80% confluency were harvested, washed in PBS and diluted 50,000 cells/mL in a total volume of 10 mL. The dilutions were set up in a 24 mL cell culture plate. 500 µL of the cells were added to one of the wells, after that 500 µL of the bacterial suspension was added to each well, mixed and transferred, in triplicate, onto a 96 well plate. The plate was then incubated for 24 hours in cell culture humidified incubator at 37˚C with 5% CO_2. After incubation, 5 µL of the cell proliferation reagent was transferred into each well. The plate was then placed on a shaker for 2 min and after 30 min incubation in the cell culture humidified chamber at 37˚C the plate was read at 450 nm on a microplate reader. % cytotoxicity was calculated using the readings of samples, normal controls and the background: % Cytotoxicity = (NC-Sample OD)/NC x 100

Controls:

NC (Negative Control): CaCo-2 cells alone

BC (Background control): DMEM alone

Calculation: 100 x (OD NC-OD sample)/OD NC

5.9. Protection of CaCo-2 cells from cytotoxicity by *L. fermetum*

Previously we saw that *L. fermentum* effectively inhibited *C. jejuni* and *C. coli* in co-culture experiments. We, therefore, were very interested in how this probiotic would behave in the infection assay. Thus we set up two experiments in parallel: in one set up we used CaCo-2 cells challenging them, in separate reactions, with *C. jejuni*, *C. coli* and *A. cryaerophilus.* In another, parallel, setup everything was identical, except that in each reaction *L. fermentum* was inoculated immediately (10 µL of OD_{600} 0.01/well) after the infection with *Arcobacter* spp. and *Campylobacter*. Following 24 H incubation and calculation of the results of each assay, we saw that the cytotoxicity effect, which ranged from 50 to 70%, in the cyctotoxicity/infection setup did not take place in the setup where *L. fermentum* was co-incubated with the *Campylobacter* isolates.

5.10. Survival of the *Arcobacter* isolates in river water

The 18 strains of *Arcobacter* were tested for their ability to survive in the river water, under refrigeration. Water was collected from the Elbe river in Magdeburg, Germany. After autoclaving and cooling the water down to room temperature, each of the 18 isolates of *Arcobacter* were first diluted to OD_{600} 0.001 in the autoclaved river water in triplicate, the dilutions corresponding to approximately 2×10^6 CFU/mL (determined separately). Then, after mixing the prepared bacterial suspensions, 10 µL of each sample was transferred, in triplicate, into 4 new sterile microfuge tubes containing 990 µL of the autoclaved river water to be plated on the same day and on days 7, 10 and 14, also in triplicate. The total count of the tubes was thus 216. After the microfuge tubes containing diluted bacterial samples were prepared and labeled, those intended for the plating on the same day (Day 1) were set aside on the bench, while the others were put away in the fridge at 4°C until needed.

For establishing the reference colony forming units (CFU) for each strain, each dilution from the triplicate was diluted further 1:10,000 in the autoclaved water, thus giving a total of 1:100,000 dilution. Then 100 µL of each sample from the triplicate diluted in this manner was plated onto a Columbia blood agar plate supplemented with sheep blood using a spreader

and plate rotation device. The plates were incubated at 37˚C for 48 h. After incubation the colonies were counted on each plate from the triplicate and the average was taken. The remaining samples were diluted and plated in the same manner on days 7, 10 and 14 with averaging the CFU counts from each triplicate. Results were then graphed using Libre Office calc version 7.4.3.2 MC OS X 12.4 (Figure 1).

5.11. Purification of bacterial genomic DNA from the *Arcobacter* and *Campylobacter* isolates

Bacterial genomic DNA was isolated by modified salting out method using 10% SDS, 5 M NaCl, proteinase K and lysozyme. Briefly, two loopfulls of bacterial colonies obtained from monoculture were resuspended in 250 µL of TES (Tris, EDTA, Sucrose) buffer using a sterile plastic inoculation loop. 50 µL of 0.01 mg/mL of lysozyme solution was added to the suspension. Following incubation at 37˚C for 30 min, 250 µL of 10% SDS was transferred into the mixture, after which 20 µL of 1 mg/mL solution of proteinase K was added. The tubes were inverted gently 3 times and then incubated at 55˚C for 30 min. After incubation, proteins and SDS were precipitated with 200 µL of 5 M NaCl. In the next step, 100 µL of preheated CTAB solution was added to the mixture, followed by a 10 min incubation at 65˚C. The tubes were then cooled off to room temperature. After adding 900 µL of chloroform-isoamyl alcohol (24:1) to the tubes, they were inverted several times and centrifuged at 15,000 RPM for 5 min. 400-500 µL of the aqueous phase was removed carefully post centrifugation and transferred to a new 2 mL microfuge tube containing 900 µL of chloroform-isoamyl alcohol mixture. The tubes were inverted again several times and centrifuged at the maximum speed of 15,000 RPM. The aqueous phase was removed without disturbing the interphase and mixed into 700 µL molecular biology grade isopropanol. DNA was precipitated by spinning the tubes at 10,000 rpm for 15 min on a benchtop refrigerated centrifuge. Pelleted DNAs were then washed with 1 mL of 70% ethanol and left to dry in a clean biosafety hood for 15 min. After 15 min 50 µL of TE buffer was added to each tube. The tubes were closed and transferred to the fridge for re-hydration overnight.

DNA concentrations were measured with Nanodrop C 100. Prior to sequencing the samples were measured once more using QUBIT kit (data not shown). The quality of purified DNA samples were checked with Agilent's Tape Station 4150.

5.12. Whole genome sequencing of the *Arcobacter* isolates

All *Arcobacter* isolates were sequenced at the Institute of Medical Microbiology and Hygiene of the OVG University of Magdeburg's School of Medicine. Illumina and Nanopore Minion platforms were used for sequencing.

Library preparation for Illumina paired-end sequencing was performed using the NEBNext® UltraTM II FS DNA Library Prep Kit for Illumina #E6177 (New England Biolabs GmbH, Frankfurt am Main, Germany).

Libraries were barcoded using the NEBNext® Multiplex Oligos for Illumina® 96 Unique Dual Index Primer Pairs #E6440S/L (New England Biolabs GmbH, Frankfurt am Main, Germany) and sequenced using the MiSeq Reagent Kit v2 (500-cycles, Illumina) as described by the manufacturer. Barcoded libraries for Nanopore long-read sequencing were prepared using the Rapid Barcoding Kit 96 (SQK-RBK110.96) according to the manufacturer's instructions and sequenced on aR9.4.1 flow cell (FLO-MIN106) on the MinION platform (Oxford Nanopore technologies ltd., Oxford, United Kingdom). Illumina paired-end reads were preprocessed using fastp (https://github.com/OpenGene/fastp, v0.23.2), and filtlong (parameters:--min_length1000--keep_percent 95, https://github.com/rrwick/Filtlong, v0.2.1) was used for long reads. Genomes were assembled unicycler v0.5.0. The assembly quality was assessed using QUAST v5.2.0. The assemblies were annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) stand-alone software version 2022-12-13.build6494. A taxonomy check was performed using mash v2.3. The full-length 16S rDNA sequences were aligned against 16S sequences of Arcobacter reference genomes using clustalW v2.1.

5.13. Phylogenetic analysis of the *Arcobacter* isolates

Phylogenetic analysis was carried out by the bioinformatics group at the Institute of Medical Microbiology and Hospital Hygiene, Otto von Guericke University School of Medicine. Phylogenetic trees were generated using IQ-TREE v2.2.2.7 or FastME v2.1.6.1, rooted at the midpoint, and visualized using Figtree v1.4.4 (https://github.com/ rambaut/figtree). Core genome analysis of respective genomes was performed using Panaroo v1.3.3. The obtained genomes were also phylogenetically analyzed using the Type (Strain) Genome Server (TYGS, accessed on 29th July 2023), which indicated the presence of a new microbial species, and in silico DNA-DNA hybridization (isDDH) was performed using the Genome-to-Genome Distance Calculator 3.0 (GGDC, http://ggdc.dsmz.de).

5.14. RAST Analysis and identification of virulence genes in the *A. tbilisiensis* genomes

RAST (Rapid Annotations using Subsystems Technology) is an automated service for annotating bacterial and archaeal genomes, which is able to identify protein-encoding, rRNA and tRNA genes. RAST assigns functions to these genes and can predict which subsystems are present in the genome. It also uses information to reconstruct the metabolic network and creates the output that is user friendly, easily accessible and downloadable. We used the following search keywords to identify virulence proteins, such as “Campylobacter”, “Virulence”, “Factor”, “Resistance”, “Drug”, and “Metabolism”.

RAST easily identified some of the virulence genes. For example, ciaB (invasion antigen) and pVIR were found in every *Arcobacter* isolate. RAST also identified a hypothetical fibronecting binding protein, the sequence of which blasted 100% to *A. cryaerophilus strain* ATCC 43158 (CP032823.1) and *A. tropharium* LMG 2534 (CP031367.1). Most of the virulence factors, however, were identified by blasting the sequences of these genes from *A. butzleri* and *A. lanthieri* reference genomes to the genomic DNA sequences of the *Arcobacter* isolates.

5.15. Determination of cytotoxicity of *C. jejuni*, *C. coli* and *A. tbilisiensis* strains on CaCo-2 cells

To evaluate cytotoxicity of the *C. coli*, *C. jejuni* and *A. tbilisiensis* isolates, Roche/Sigma Aldrich WST1 cell proliferation reagent (Sigma Aldrich, Taufkirchen, Germany) and CaCo-2 cells were used. For this purpose, CaCo-2 cells were maintained in cell culture in DMEM supplemented with 2% FBS. All *Arcobacter* isolates and some *C. coli and C. jejuni* isolates were used in a cytotoxicity study (Tables 6 and 7). Prior to the day of the experiment, each bacterial strain was re-inoculated on Columbia blood agar (Oxoid, Wessel, Germany). After 24 hours, a medium-sized colony was resuspended from each plate into 1 mL of DMEM in a sterile microfuge tube and then diluted further to OD_{600} 0.01. CaCo-2 cells grown to 80% confluency were harvested, washed in PBS and diluted 50,000 cells/mL in a total volume of 10 mL. The dilutions were set up in a 24 mL cell culture plate. 500 µL of the cells were added to one of the wells, after that 500 µL of the bacterial suspension was added to each well, mixed and transferred, in triplicate, onto a 96 well plate. The plate was then incubated for 24 hours in cell culture humidified incubator at 37°C with 5% CO_2. After incubation, 10 µL of the cell proliferation reagent/well was used. After 2 hour incubation of the plate in the cell culture humidified chamber at 37°C the plate was read at 450 nm on a microplate reader. Then percent cytotoxicity was calculated using the readings of samples, normal controls and the background.

6 Results

6.1. Sample collection

6.1.1 Isolation of *Arcobacter spp.* and *Campylobacter* spp. from retail chicken meat

18 isolates of *Arcobacter*, 39 isolates of *C. jejuni* and 35 isolates of *C. coli* were cultured from various chicken meats bought in supermarkets in Tbilisi. The species identification of the isolates was performed using MALDI TOF mass spectrometry (Biomérieux, Nürtingen,

Germany) at the Institute of Medical Microbiology and Hospital Hygiene, Medical Faculty, Otto-von-Guericke University Magdeburg, Magdeburg, Germany.

6.1.2 Species identification of the *Campylobacter* and *Arcobacter* isolates by MALDI-TOF MS

Out of 107 total isolates 93 were confirmed as either *Campylobacter* or *Arcobacter* as follows:

ID-ed Species	**N of Isolates**
C. jejuni	39 isolates
C. coli	35 isolates
A. cryaerophilus	19 isolates

One of the LAB isolates that demonstrated activity against *Campylobacter* spp. and *Arcobacter* spp. was also ID-ed by Mass Spectrometry as *L. fermentum.*

Notably, *Arcobacter* isolates were first identified as *A. cryaerophilus* based on MALDI TOF Mass Spectrometry. However, whole genome sequencing analysis later showed significant divergence from *A. cryaerophilus* and other existing *Arcobacter* reference strains, e.g. *A. butzleri*, *A. lanthieri*, *A. tropharium* etc. Thus, based on genome and proteome analysis, we encountered enough evidence of a novel species of *Arcobacter: Arcobacter tbilisiensis*. sp. nov. This will be discussed further in the sequencing and phlylogenetic analysis sections.

MALDI-TOF MS analyses of *A. cryaerophilus* ATCC 43158 and *A. tbilisiensis* sp. nov. strain 51/LEO 51 (as well as the rest of 18 isolates included in the study) were conducted. All isolates were cultured on Mueller Hinton Agar supplemented with horse blood (MHF, because A. cryaerophilus ATCC 43158 grows only on MHF-Agar and not on Columbia sheep blood agar) under microaerophilic conditions in the same jar prior to mass spectrometry. For analysis, 600 spectra from 2-20 kDa were gathered in 100-shots steps and added. The MALDI-Biotyper identification for both microbial species was determined to be *A. cryaerophilus*, with the crucial difference being that repeated measurements of *A. cryaerophilus* ATCC 43158 Biotyper identification score values reached 2.1 to 2.2, while the

Biotyper identification score values of the *A. tbilisiensis* sp. nov. isolates ranged from 1.8 to 1.9. Typically, Biotyper identification score values ≥2.000 are considered reliable for microbial species identification. Therefore, the identification of the *A. tbilisiensis* sp. nov. isolates as *A. cryaerophilus* based on the score values is not reliable. In an exploratory assessment of the overlaid intact cell MALDI-TOF mass spectrometry (ICMS) generated representative spectra (Fig. 5) of the designated type strain *A. tbilisiensis* sp. nov. LEO 51 (DSM 115960) and *A. cryaerophilus* type strain ATCC 43158 (DSM 7289), in addition to allelic isoform-related mass shifts of biomarker ions present in both microbial species, species-specific biomarker ions were also observed. As shown in Fig. 1, several biomarker ions specific for *A. tbilisiensis* sp. nov. were detected e.g. at m/z values of 7,562.48; 8,011.02; 11,463.53; 12,688.00; 13,253.18; 15,113.40; 16,019.56; and 16,608.02. In contrast an *A. cryaerophilus* specific biomarker ion was observable at m/z = 13,541.49. Due to the significant number of species-specific biomarker ions, the unambiguous identification of this microbial species by MALDI-TOF MS should be possible after depositing the main spectrum peaks (MSPs) in the reference database.

Overlay of intact cell MALDI-TOF mass spectrometry (ICMS) generated representative spectra of *A. tbilisiensis* LEO 51 (DSM 115960, red) and *A. cryaerophilus* type strain ATCC 43158 (DSM 7289, blue). Biomarker ions that are specific to *A. tbilisiensis* sp. nov. and thus distinguish it from the most closely related microbial species, *A. cryaerophilus,* have been highlighted with red arrows.

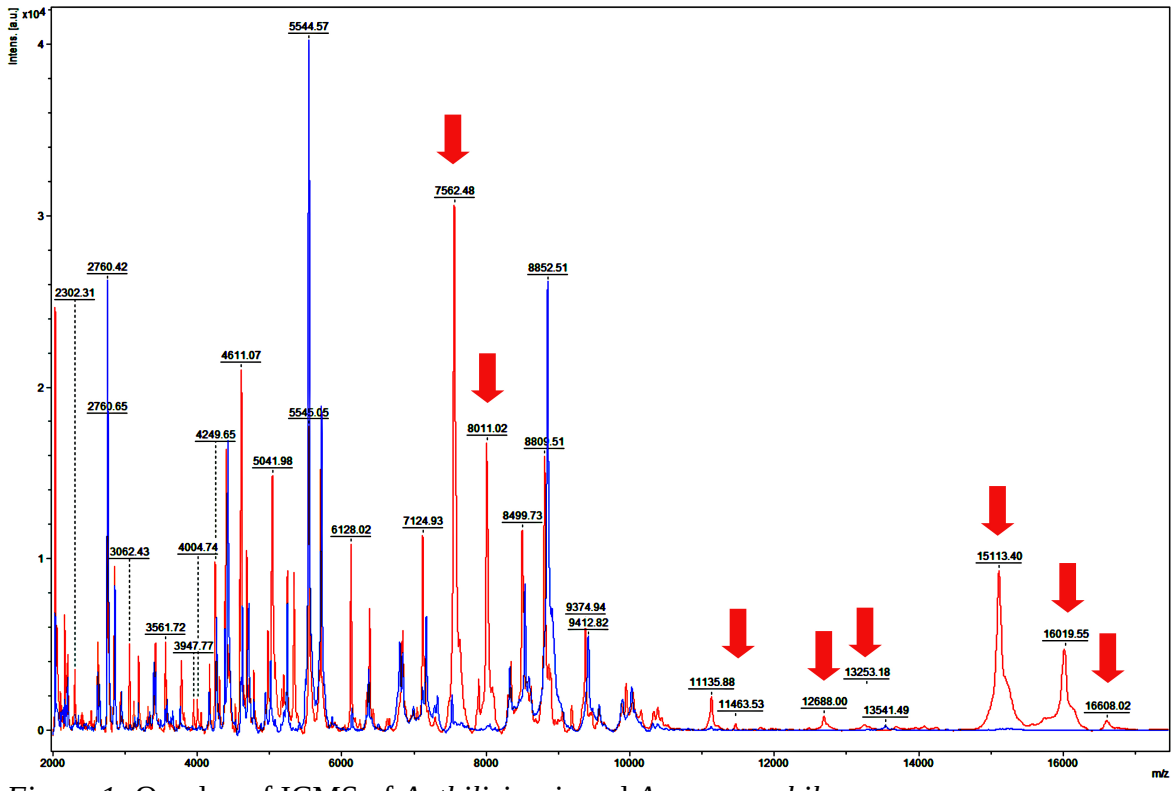

Figure 1: Overlay of ICMS of *A. tbilisiensis* and *A. cryaerophilus*

6.2. Conventional light microscopy

6.2.1 *Campylobacter* spp.

Staining of *Campylobacter* spp. revealed Gram-negative curved rods. The bacteria were pleomorphic, most having "S", "corkscrew" and "comma" shapes. Occasionally "serpent" shape was also noted, but we never observed the classical "seagull" shape.

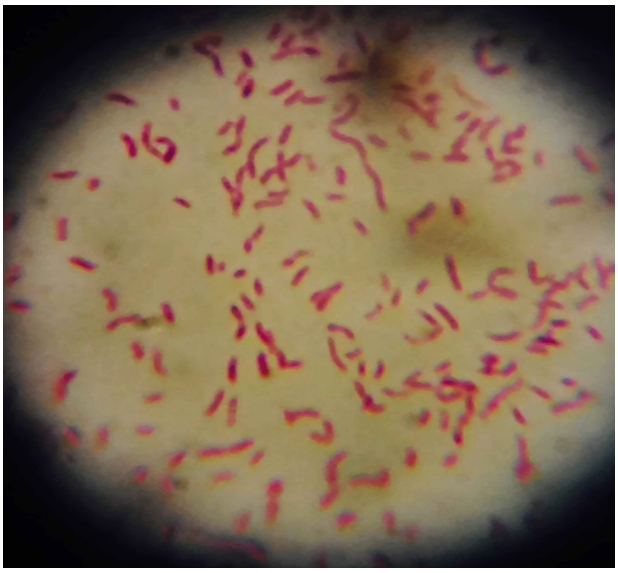

Figure 2: *C. jejuni* stained w. carbol fuchsin

Figure 3: *C. jejuni* on CCDA Agar Plate

6.2.2 Identification of a Novel *Arcobacter* spp. *A. tbilisiensis* sp. nov.

We observed Arcobacter spp. as much finer and thinner pleomorphic rods in gram stains, compared to *Campylobacter* spp. Originally we assumed these were also some *Campylobacter* species, until Mass Spectrometry and Next Generation Sequencing determined them to be *Arcobacter* spp.

Figure 4: *A. tbilisiensis* sp. nov. on COS agar

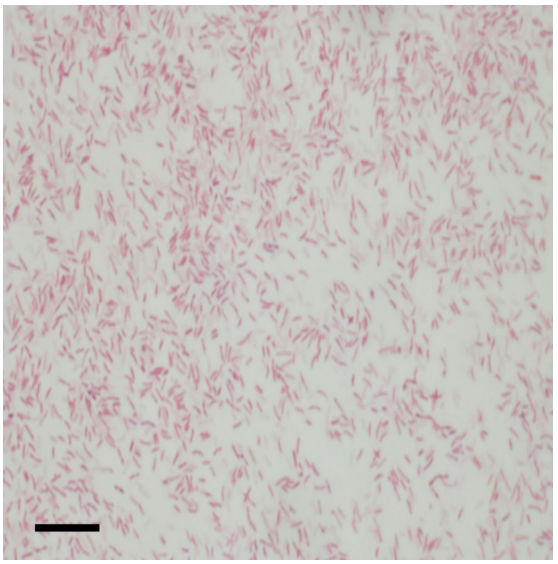

Figure 5: *A. tbilisiensis* sp. nov. Gram stain

Arcobacter spp. were isolated by the same method as *Campylobacter* spp. At the beginning of the research project little phenotypic differences were noted between the different isolates of *Campylobacter* spp. and *Arcobacter* spp. For example, we observed that *C. jejun*i tends to *p*roduce larger, elongated and off-white colonies resembling water droplets, while *C. coli* colonies were smaller and grayish-tan. *Arcobacter* colonies, on the other hand, were very small, more flat than concave and looked translucent against the black background. Strains were obtained from the samples intended for *Campylobacter* isolation, therefore culture plates were incubated at 37˚C under microaerophilic conditions.

6.3. Sequencing of genomic DNAs of the *Arcobacter* isolates by NGS

All 19 Arcobacter DNAs were sequenced using Illumina (San Diego, CA, USA) and Minion (Oxford Nanopore, Oxford, Great Britain) technologies and sequences were uploaded as text files on a compact disc, which is supplemented to this PhD thesis. Each Arcobacter isolate consists of one contig, except for the three strains (51, 62 and 65) that harbor plasmids. Strains 103 and 107 harbor 2 plasmids. The genomic DNA size of the isolates is over 2 million base pairs (e.g. for isolate 46 it is 2,136,238 bp).

Figure 6. Doughnut-Blot of the percentage distribution of the COG categories to which individual CDSs in the genome of Arcobacter tbilisiensis LEO 51 (DSM 115960) was assigned. The 20 most abundant categories level identified by EggNOG 5.0 are represented by a specific color.

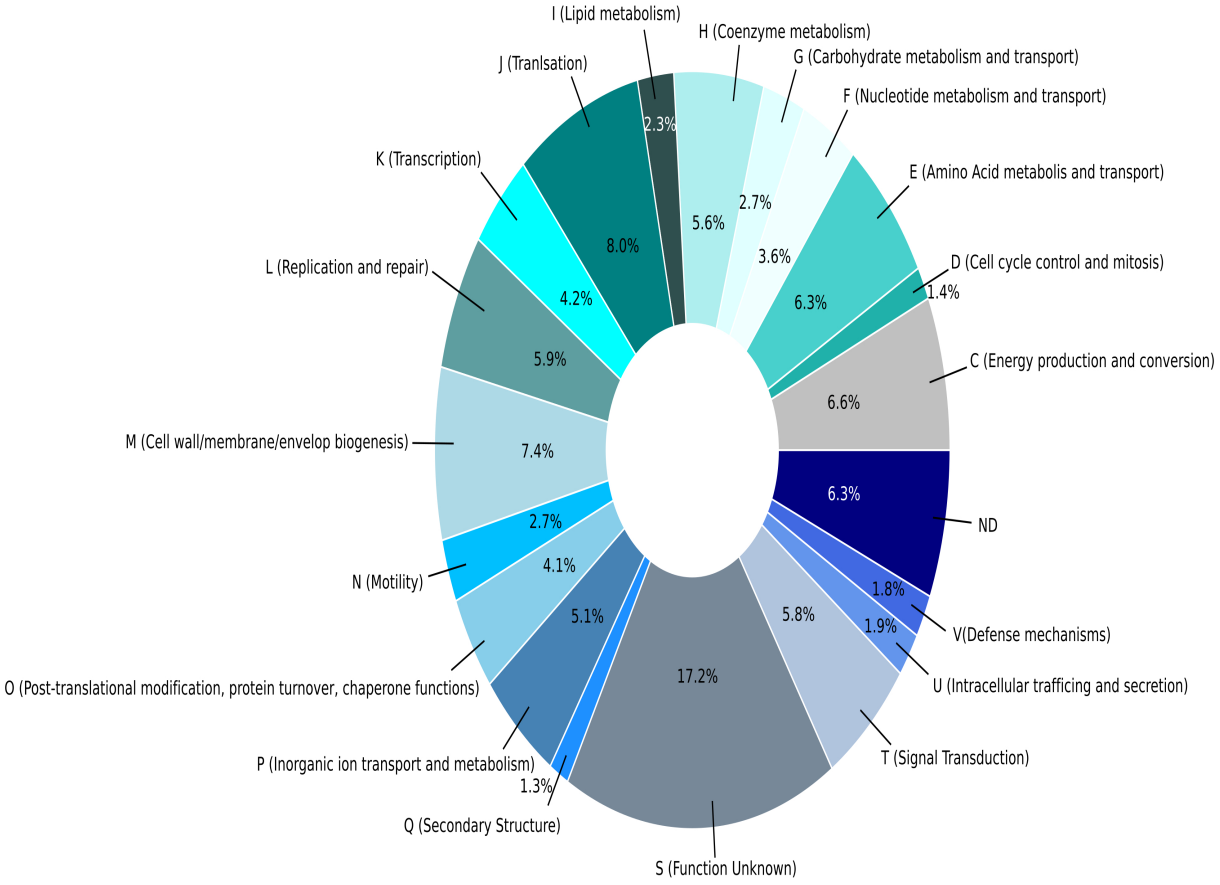

Figure 6: Dougnut-Blot of percent distribution of the COG categories

6.4. Phylogenetic analysis of *Arcobacter* genome

Phlylogenetic analyses are based on 16S RNA and whole genome DNA sequences. As a result of this analysis, the *Arcobacter* isolates identified in this study showed significant divergence from the existing reference genomes in the NCBI database.

Figure 7. Root phylogenetic tree inferred from Genome Blast Distance Phylogeny (GBDP) distances calculated from 16S rRNA gene sequences. The branch lengths are scaled in terms of GBDP distance formula d5. The numbers above branches are GBDP pseudo-bootstrap support values > 60 % from 100.

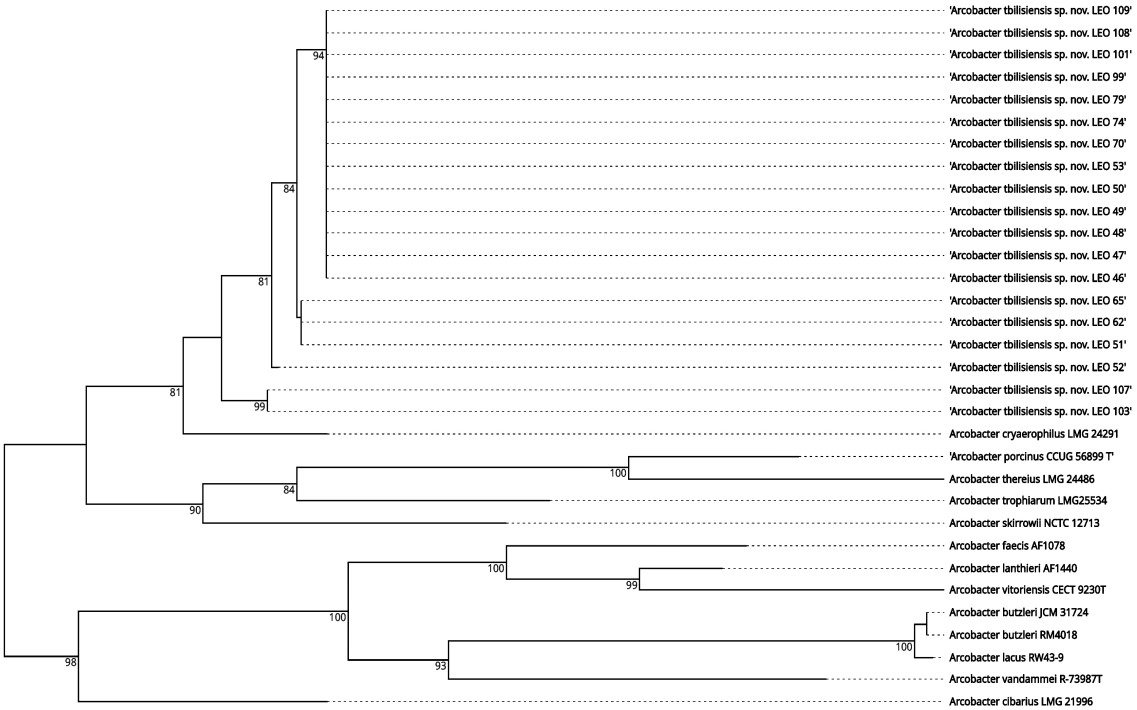

Figure 7: Root phylogenetic tree

Figure 8. Midpoint rooted phylogenetic tree inferred from Genome Blast Distance Phylogeny (GBDP) distances calculated from genome sequences. The branch lengths are scaled in terms of GBDP distance formula d5. The numbers above branches are GBDP pseudo-bootstrap support values > 60% from 100 replications, with an average branch support of 46.9%.

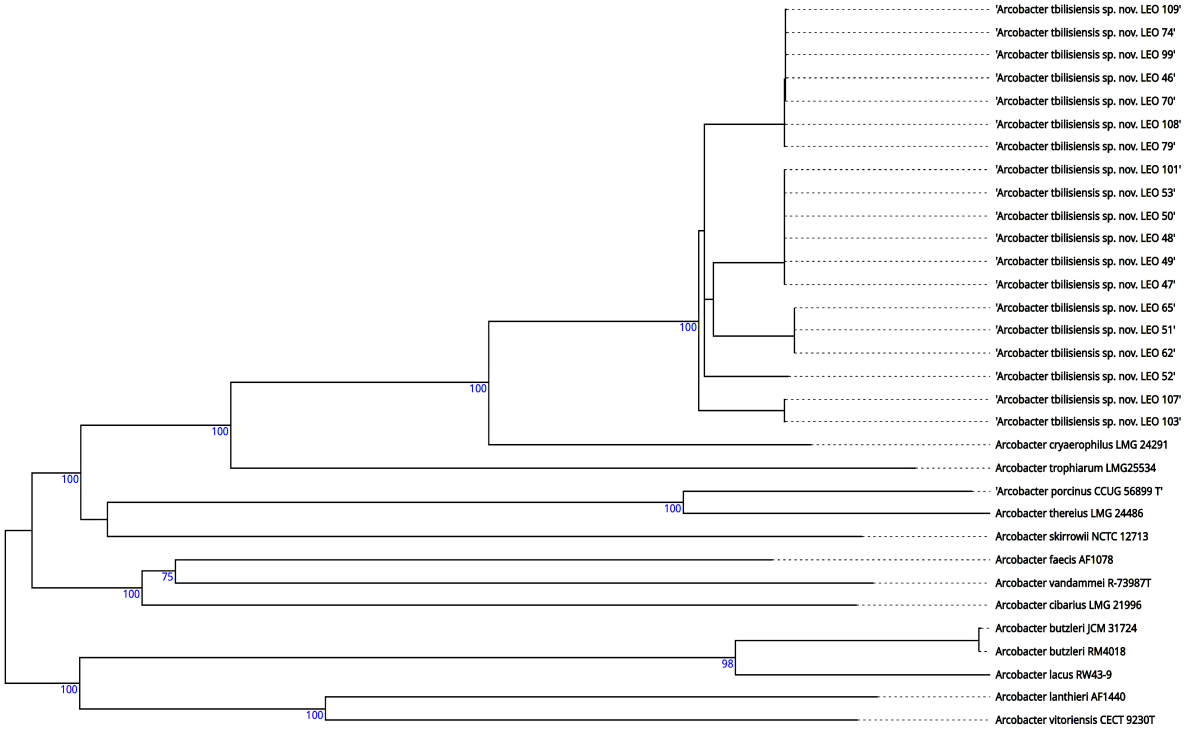

Figure 8 Midpoint root phylogenetic tree

Figure 9. Midpoint-rooted maximum-likelihood tree from (A, left) ClustalW alignments of BLASTn results for *Arcobacter tbilisiensis* LEO103 (DSM 115972), LEO51 (DSM 115960), and LEO46 (DSM 115954) full length 16S rDNA against NCBI 16S ribosomal RNA sequence database and from (B, right) Mafft alignments of 66 core-genes as determined by Panaroo. In both dendrograms the representatives are referred to as genomovars of *A. cryaerophilus*: *"A. cryaerophilus* gv. *pseudocryaerophilus*" (LMG 10229), *"A. cryaerophilus* gv. *crypticus*" (LMG 9065), "*A. cryaerophilus* gv. *cryaerophilus*" (LMG 24291) and "*A. cryaerophilus* gv. *occultus*" (LMG 29976).

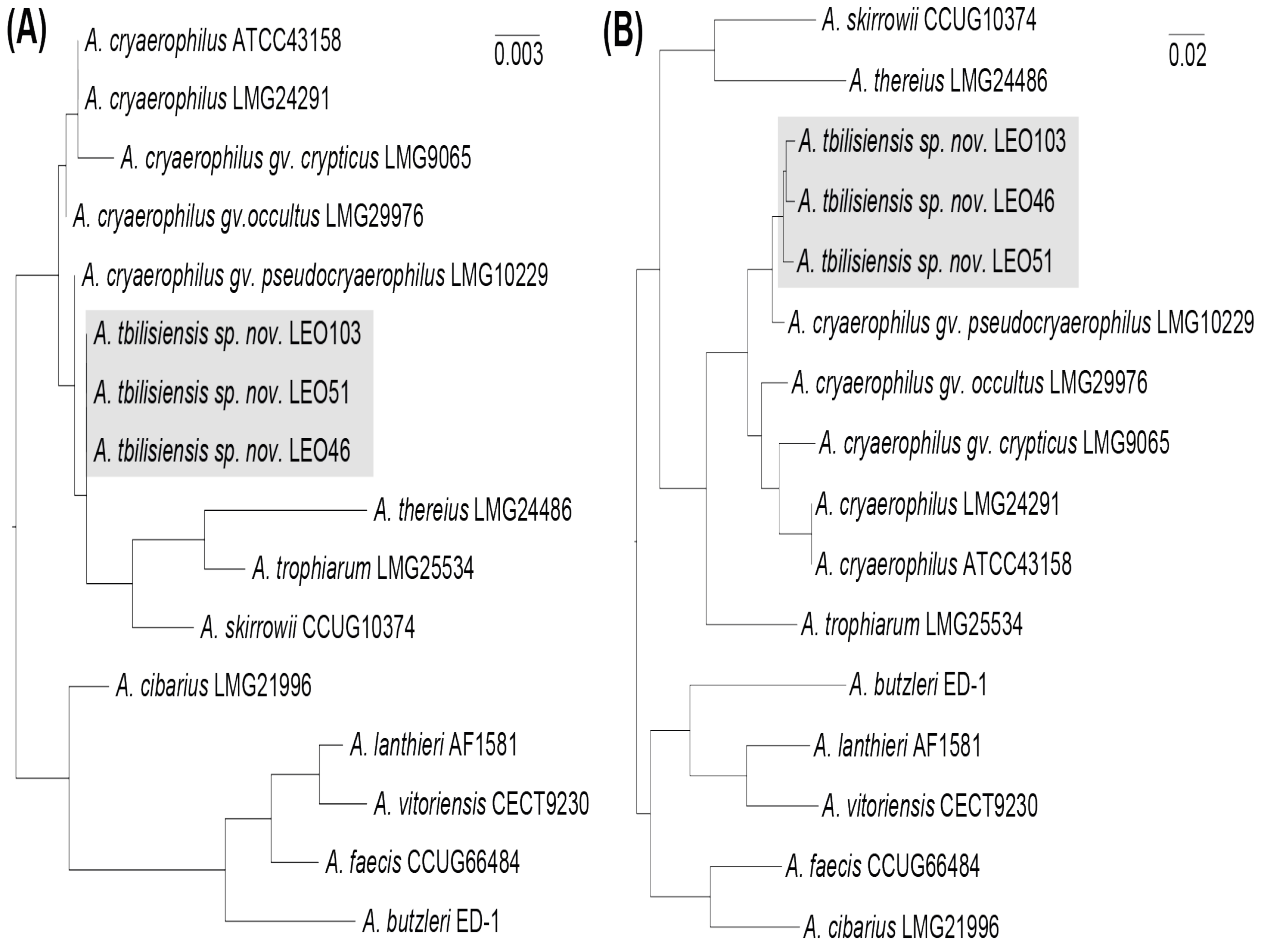

Figure 9 Midpoint rooted maximum likelihood tree

6.5. API testing of *A. tbilisiensis* sp. nov.

API®CAMPY test was performed on the three *A. tbilisiensis isolates* (46, 51 and 103) as part of biochemical testing of the novel species.

Table 3. Results of the API®CAMPY test strips for the three *Arcobacter tbilisiensis* sp. nov. isolates LEO 46 (DSM 115954), LEO 51 (DSM 115960), and LEO 103 (DSM 115972) representing three different phylogenetic clades.

Characteristic	LEO 46 DSM 115954	LEO 51 DSM 115960	LEO 103 DSM 115972
Gram staining	-	-	-
Motility	+	+	+
Urease	-	-	-
Reduction of nitrates to nitrites	+	+	+
Esterase	+	+	+
Hippurate hydrolysis	-	-	-
Gamma-glutamyl-transferase	-	-	-
Reduction of triphenyltetrazoliumchloride	+	+	(+)
Pyrrolidonyl-arylamidase	-	-	-
L-arginin-arylamidase	-	-	-
L-aspartate-arylamidase	-	-	-
Alkaline phosphatase	+	+	+
H_2S production	-	-	-
Catalase	+	+	+
Oxidase	+	+	+
Glucose assimilation	-	-	-

Succinate assimilation	-	-	-
Nalidixic acid (growth inhibition)	susceptible	susceptible	susceptible
Cefazolin (growth inhibition)	susceptible	susceptible	susceptible
Acetate assimilation	+	+	+
Proprionate assimilation	-	-	-
Malate assimilation	-	-	-
Citrate assimilation	-	-	-
Erythromycin (growth inhibition)	susceptible	susceptible	Susceptible

6.6. Identification of virulence genes of *A. tbilisiensis* sp. nov. isolates

Some virulence-associated factors encoding genes such as ciaB, as well as various other virulence factors, such as proteins involved in oxidative stress, were identified by RAST. However the virulence factors listed in the table below were identified by blasting the sequences of the specific genes of *A. butzleri* and *A. lanthieri* reference strains available on NCBI.

Table 3. Virulence factors identified in all *A. tbilisiensis i*solates

Gene	Definition	Quiery ID	Species	Quiery Start	Query End	Subject Start	Subject End	% Coverage	% Identity
cadF	Campylobacter Adhesion Factor/fibronecting binding protein	MG434461.1	*A. lanthieri* LMG:28516	1	1017	674221	675240	100	77.9
cdtA	Cytolethal distending toxin A	MG434467.1	*A. lanthieri* LMG:28516	1	1825	1845741	1863920	99	83.01
cdtB	Cytolethal distending toxin B	MG434468.1	*A. lanthieri* LMG:28516	1	1290	387975	389267	100	80.4
cdtC	Cytolethal distending toxin C	MG434469.1	*A. lanthieri* LMG:28516	76	1632	46216	44666	92	82.98
ciaB	Campylobacter Invasion Antigen B	LC581320.1	*A. butzleri* BON-Jun-27	1	969	755022	756000	96	82.03
CJ 1349	fibrinogen/fibronectin binding protein	HF935058.1	*A. butzleri*, strain F1	10	537	974130	974656	98	71.83
irgA	Iron regµLated outer membrane protein	LLKQ01000001.1	*A. thereius LMG 24486 AA347*	1478596	1479376	205293	206070	45	72.14
mviN	Murein virulence	NREO01000016.1	*A. suis* CECT7833	23002	302231	2106026	2113172	80	74.59
pldA	Phospholipase	MG434465.1	*A. lanthieri* LMG 25816	9	936	205136	20630	99	74.57
tlyA	Mycobacterial hemolysis factor	CP053833.1	*A. cloacae* LMG 26153	2133610	2134319	1648364	1647646	99	75.69

6.7. Antibiotic susceptibility testing results of *A. tbilisiensis* isolates

Table 4. Summary of antimicrobial resistance of the *Arcobacter* isolates

Class	Antibiotic	Cutoff	Resistant Strains
Penicillins	Penicillin G	>15 mm	All
	Ampicillin	>13 mm	**44.4%**
Aminoglycosides	Streptomycin		None
	Kanamycin	>13 mm	None
	Gentamicin	>19 mm	**11%**
Macrolides	Chloramphenicol	>18	None
	Erythromycin	>13	None
Fluoroquinolones	Ciprofloxacin	>17 mm	**22%**
Tetracyclines	Tetracycline	>17 mm	None

We thus identified that some *A. tbilisiensis* sp. nov. strains as resistant to 4 different groups of antibiotics: penicillins, fluoroquinolones, macrolides and aminoglycosides.

6.8. Antibiotic susceptibility testing results of *C. jejuni* and *C. coli*

Table 5. Summary of antimicrobial resistance of *C. jejuni* and *C. coli* isolates

Class	Antibiotic	Cutoff	Campylobacter Species	**Resistant Isolates**
Penicillins	Penicillin G	>15 mm	*C. coli*	All
			C. jejuni	All
	Ampicillin	>13 mm	*C. coli*	**51.43%**
			C. jejuni	**28.21%**
Aminoglycosides	Streptomycin	>11 mm	*C. coli*	**2.86%**
			C. jejuni	**2.56%**
	Kanamycin	>13 mm	*C. coli*	None
			C. jejuni	None
	Gentamicin	>19 mm	*C. coli*	None
			C. jejuni	None
Macrolides	Chloramphenicol	>18 mm	*C. coli*	**2.86%**
			C. jejuni	None
	Erythromycin	>20-<20	*C. coli*	None
			C. jejuni	None
Fluoroquinolones	Ciprofloxacin	>50-<26 mm	*C. coli*	**97.14**%
			C. jejuni	**79.49**%
Tetracyclines	Tetracycline	>30 -<30 mm	*C. coli*	**51.43**%
			C. jejuni	**28.21**%

The highest resistance observed among the isolates of *C. jejuni* and *C. coli* were to ciprofloxacin (79.49% and 97.14%, respectively), tetracycline (28.21% and 51.43%, respectively) and ampicillin (28.21% and 51.43%, respectively).

6.9. Activity of *L. fermentum* against *Campylobacter* spp. in co-incubation assay

Several strains of LAB were isolated in our laboratory from various products, such as fermented cabbage, fermented milk, and fermented cucumbers. In this manner we isolated several strains of *L. plantarum,* one strain of *L. paracasei* and a strain of *L. fermentum.* The identities of these isolates were determined using MALDI-TOF mass spectrometer in Magdeburg, Germany. Out of all tested LAB species, we identified one strain of *Lactobacillus fermentum* that demonstrated inhibitory activity against *Campylobacter* spp. Co-incubation experiments of *L. fermentum* with *C. jejuni* and *C. coli* isolates resulted in the effective inhibition of *C. jejuni* 003 and *C. coli* 002. While in all other setups with different lactobacilli *Campylobacter* spp. grew well in places where they were spotted on a CCDA plate in triplicate, co-incubation with *L. fermentum* resulted in a complete absence of growth.

Because *Campylobacter* spp. are known to form biofilms and because 0.9 OD_{600} contains billions of CFU, we reasoned that 0.2 OD_{600} would be a good number, based on the data that 0.2 OD_{600} corresponds to 10^9 bacterial counts/mL (Stingl et al., 2021). Refer to the figures below for the results.

Position	Setup
Row 1:	NC (*C. jejuni* 003)
Row 2:	*L. rhamnosus 51152 + C. jejuni 003*
Row 3:	*L. fermentum + C. jejuni 003*
Row 4:	*L. delbrueckii 510060 + C. jejuni 003*

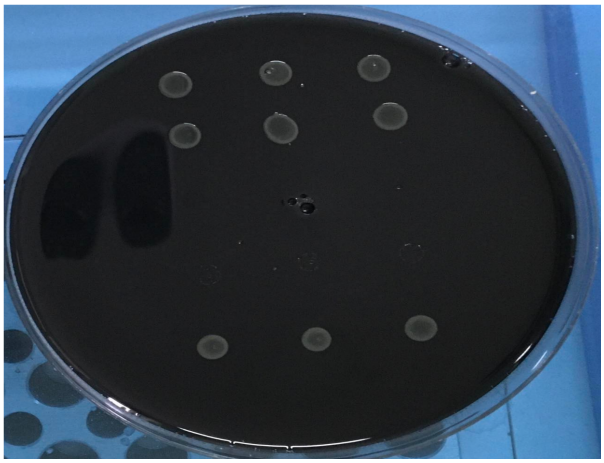

Figure 10: Inhibitory Effect of *L. fermentum* on *C. jejuni*

Position	Setup
Row 1:	NC (*C. coli* 002)
Row 2:	*L. rhamnosus 51152 + C. coli 002*
Row 3:	*L. fermentum + C. coli 002*
Row 4:	*L. delbrueckii 510060 + C. coli 002*

Figure 11: Inhibitory Effect of *L. fermentum* on *C. coli*

6.10. Determination of cytotoxicity of *C. jejuni*, *C. coli* and *A. tbilisiensis* sp. nov. strains

Cytotoxicity results revealed that all *A. tbilisiensis* sp. nov. isolates are characterized with significant cytotoxicity ranging from 52% to 87% (refer to Table 6). A very encouraging observation was that infection of CaCo-2 cells with *Arcobacter* isolates in the presence of *L. fermentum* resulted in complete amelioration of cytotoxicity (refer to Table 7).

Table 6. Cytotoxic effect of *Arcobacter* isolates on CaCo-2 Cells

A. tbilisiensis Isolate	Average Final OD	% Cytotoxicity
	NC average=2.81	
46	0.45	83
47	0.51	82
48	0.61	78
49	0.55	64
50	0.59	79
51	0.5	79
52	0.48	83
53	0.49	82
62	0.54	80
65	0.62	78
70	0.65	52
79	0.47	83
99	0.55	78
101	0.6	79
103	0.37	87
107	0.39	86
108	0.63	78

Table 7. Cytotoxic effect of *C. jejuni*, *C. coli* and *A. tbilisiensis* isolates on CaCo-2 cells with and without the presence of *L. fermentum*

Isolate	Without *L. fermentum* OD	StDev	CV	% Cytotox.	Control Average
115 (*C. coli*)	0.47	0.21	14.69	83	2.72
99 (*A. tbilisiensis*)	0.52	0.17	13.39	81	
38 (*C. jejuni*)	0.45	0.11	6.17	83	
104 (*C. jejuni*)	0.38	0.06	4.93	86	
105 (*C. coli*)	0.4	0.1	5.46	85	
106 (*C. coli*)	0.53	0.2	10.09	81	
Isolate	**With *L. fermentum***			**% Cytotox.**	**Control Average**
115 (*C. coli*)	2.7	0.01	3.27	-0.7	2. 72
99 (*A. tbilisiensis*)	2.67	0.01	2.55	1.8	
38 (*C. jejuni*)	3.11	0.05	11.4	-14.3	
104 (*C. jejuni*)	2.82	0.02	6.38	-3.6	
105 (*C. coli*)	2.75	0.03	6.19	-1.1	
	3.24	0.02	5.07	-19.11	

6.11. Survival of *A. tbilisiensis* in river water under refrigeration

The 18 isolates of *Arcobacter,* generally, had the same survival rate with all of the strains showing little to no difference in the number of CFUs between days 1 and 7 the medians are very close for both days (202 for day 1 and 198 for day 7). The median count is the lowest on day 10 equalling 17. Only one strain out of 18 produced colonies after day 14. These data imply that *A. tbilisiensis* can survive refrigeration for at least 10 days. However, taking into

consideration of the fact that chicken juice present in the packaging contains nutrients, this pathogen can in fact survive for longer periods, as contaminant.

Table 8. Survival of the *Arcobacter tbilisiensis* isolates in river water under refrigeration CFU count Results from Days 1, 7, 10 and 14.

A. tbilisiensis Strain	Day 1	Day 7	Day 10	Day 14
“101”	373	351	271	0
“50”	362	315	57	0
“58”	332	301	56	0
“51”	318	298	48	0
“46”	318	297	37	0
“74”	305	296	32	0
“52”	296	291	32	0
“62”	275	213	31	0
“79”	271	205	26	0
“53”	256	201	21	0
“49”	202	198	17	0
“103”	180	182	15	0
“95”	156	111	8	0
“48”	155	105	6	0
“99”	142	97	5	0
“47”	120	82	3	0
“65”	114	0	2	0
“107”	105	0	0	0

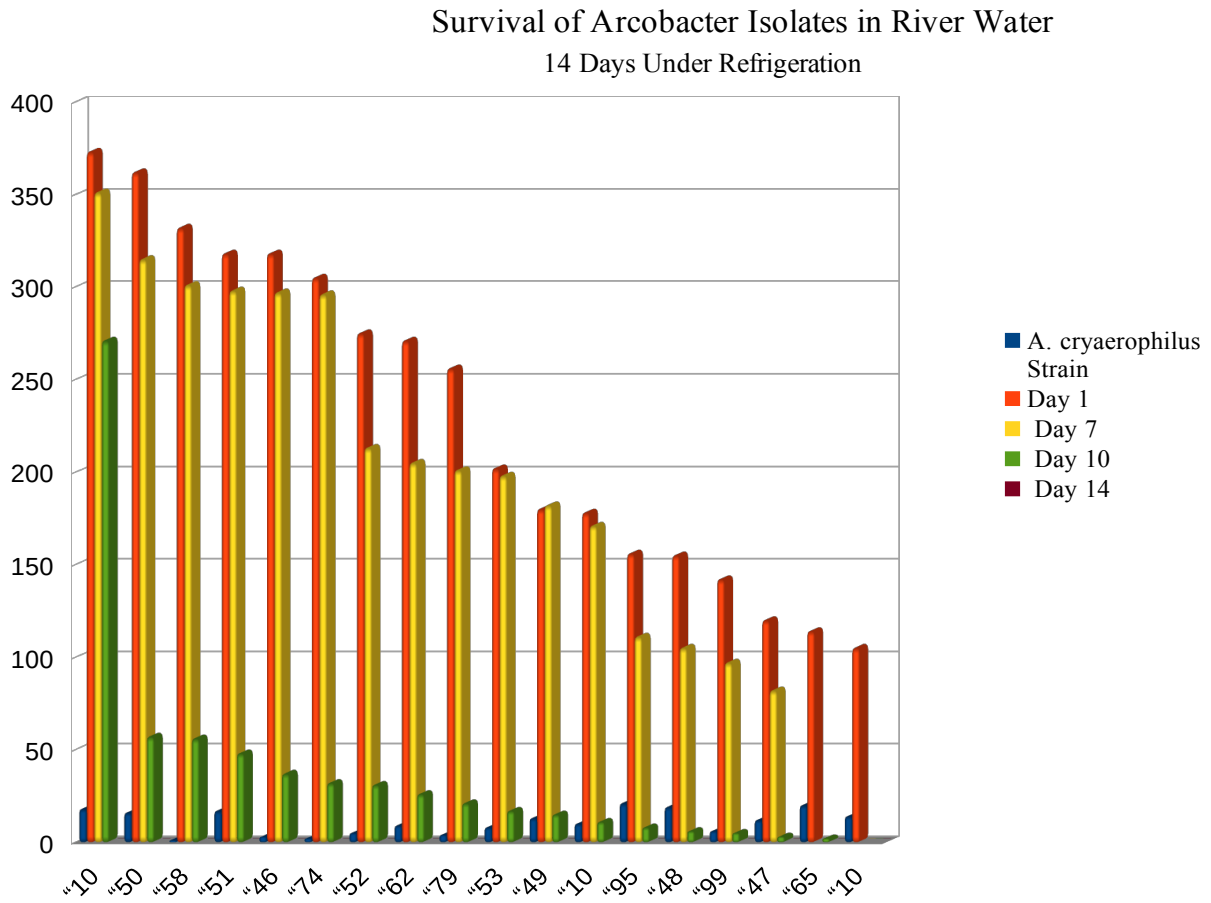

Figure 12: Survival of Arcobacter isolates in river water

While practically the same CFUs were isolated on days 1 and 7, day 10 resulted in a dramatic decline of the CFU counts. By day 14 only one isolate remained culturable.

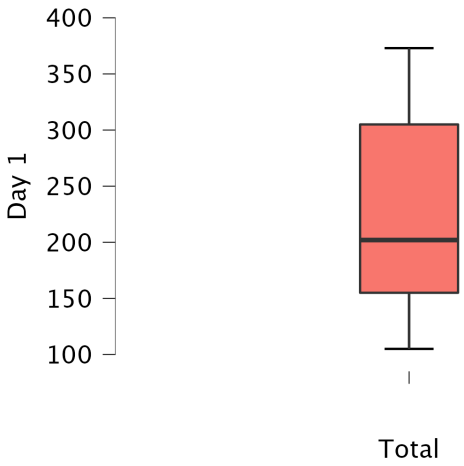

Figure 13*:* Water Survival, Day 1, Median

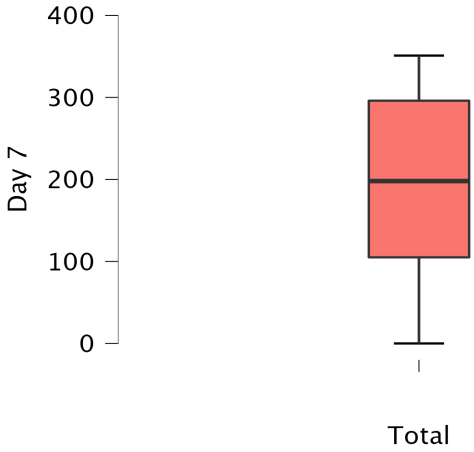

Figure 14*:* Water Survival, Day 7, Median

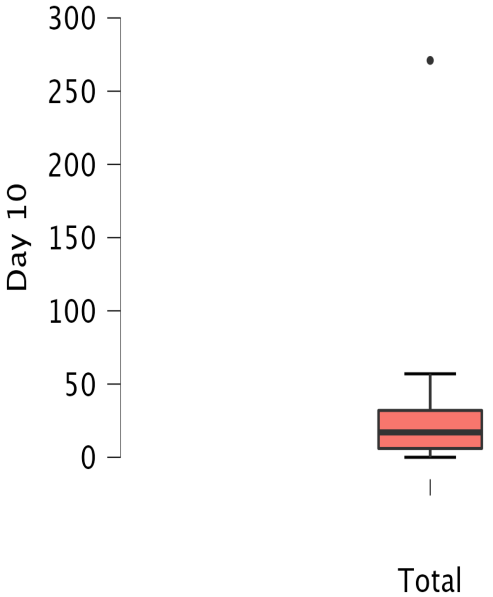

Figure 15*:* Water Survival, Day 10, Median

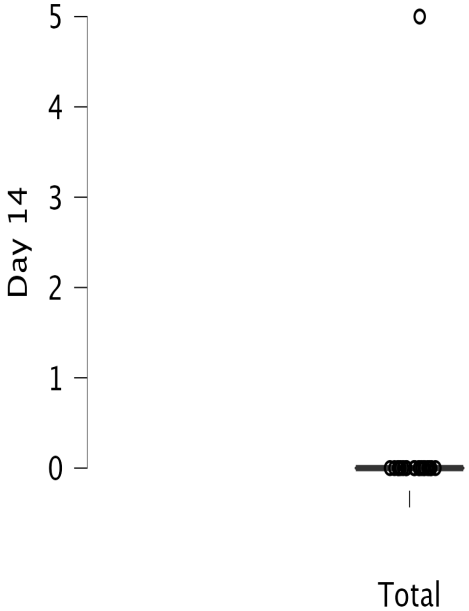

Figure 16*:* Water Survival, day 14, Median

7 Discussion

This is the first example of isolation and characterization of *Arcobacter* and *Campylobacter* spp. from any meat source produced in Georgia. These significant findings warrant more, larger-scale studies in the future.

7.1. Isolation of *Arcobacter* spp. and *Campylobacter* spp.

Generally, methods of isolating of *Campylobacter* spp. and *Arcobacter* spp. often require expensive reagents, such as microaerobic gas pouches, enriched media etc. Because our budget was limited as to what reagents and equipment we could purchase, we decided to focus on previous studies using relatively affordable protocols. Therefore, in this regard the isolation method described by Oyarzabal et al. was convenient (Oyarzabal et al., 2013). To create microaerobic atmosphere we used short candles, sometimes two, per jar. CCDA seemed like the best choice for *Campylobacter* isolation, since in the previous attempt using Preston agar proved to be ineffective in our hands. Once isolated, both *Arcobacter and Campylobacter* spp. can easily be cultured on Columbia Blood Agar or Mueller Hinton agar supplemented with sheep blood. The second important factor was the choice of antibiotic supplements. From our experience, the problem with isolation with either *Campylobacter* or *Arcobacter* from chicken meats is that a lot of other contaminant species-such as *Pseudomonas* spp., *Orthobacter* spp. and others can overgrow the plate because Campylobacter is a slow grower. Thus we purchased Campylobacter antibiotic supplement with cefoperazone and amphotericin B, which worked very well. Generally, pre-incubation of meat samples in peptone water or bolton broth for 1-2 hours prior to inoculating plates was effective. However, we did not find much difference between the two options. Another effective finding was to inoculate multiple CCDA plates, rather than one or two. This, as well as incubating plates at 37˚C post inoculation rather than at 42˚C dramatically improved isolation.

We suspect that *Arcobacter* prevalence would be higher in Georgian raw chicken meat if we used *Arcobacter*-specific enrichment.

7.2. Purification and sequencing of bacterial genomic DNAs

We did not encounter any problems in isolating good quality DNA, as the DNA purification procedure was based on classical salting out method with some modifications. All DNA concentrations and 260/280 and 260/230 ratios (data not shown) were satisfactory for NGS (Next Generation Sequencing).

7.3. MALDI-TOF Mass Spectrometry

Campylobacter isolates were identified as either *C. jejuni* or *C. coli. Arcobacter* isolates were first identified as *A. cryaerophilus* by the same method of matrix laser desorption. However, phylogenetic analysis later showed that the isolates belonged to a new *Arcobacter* species: *A. tbilisiensis* sp. nov.

Identification of bacteria by MALDI-TOF MS relies on existing spectral databases. For example, to properly identify a bacterial species, the database must contain information about specific genes, such as the 16S rRNA gene, gyrB, rpoB, or hsp60 of strains/species of a particular genus. For certain taxa, such as *Streptococcus* or *Staphylococcus* geographical variations often lead to differences in the genotype and phenotype. Therefore, for such taxa locally prepared databases should be used (Singhal et al. 2015; Rychert, 2019).

7.4. Phylogenetic analysis of the *Arcobacter* isolates

Phylogenetic analysis placed *A. tbilisiensis* sp. nov. as a species diverging from *A. cryaerophilus,* and forming 5 different clades. We did not find any clade-specific antibiotic susceptibility patterns. Naturally, differentiation of the *A. tbilisiensis* isolates into different clades must be due to differences in the genomes of these closely related members of the novel species. Similarly, differences between 1A and 1B types of *A. cryaerophilus* could also be detected by MALDI-TOF MS (Pérez-Cataluña et al., 2018). The phylogenetic analysis of *A. tbilisiensis* strains was also based on the 16S rRNA gene sequence, which, along with 23S rRNA gene sequence, served as the basis for the division of *A. cryaerophilus* into 1A and 1B subgroups represented by strains LMG 24291 and LMG 10829 (Vandamme et al., 1991).

7.5. Antibiotic susceptibility among the isolates of *A. tbilisiensis*, sp. nov. *C. jejuni and C. coli*

Antibiotic susceptibility testing results of the *A. tbilisiensis* sp. nov. isolates agree with those identified by various research groups all over the world. Even though sub-therapeutic use of antibiotics in the EU has been banned, antimicrobials are till being used in the United States and many developing countries. Thus, ciprofloxacin and tetracycline resistance among the Georgian *Campylobacter* isolates is high and comparable to the reports from studies conducted many other developing countries.

From the 19 isolates of *A. tbilisiensis* all were susceptible to erythromycin, streptomycin, chloramphenicol, kanamycin and tetracycline. Susceptibility to the latter is surprising, because we found high resistance levels to tetracycline among the *Campylobacter* isolates. Susceptibility of *Arcobacter* spp., e.g. *A. skirowii,* to tetracycline, have been reported by other studies as well (Hänel et al. 2021). An earlier, 2011 Turkish study of 70 *A. butzleri* strains isolated from various meats and symptomatic humans identified that 2.85% of the isolates as resistant to tetracyclin, thus recommending this drug for treating animals and humans (Abay et al. 2012). However, Oliveira et al., and other researchers have reported resistance to tetracycline in *Arcobacter* spp. isolated from chicken meat (S. Yesilmen et al. 2017; Oliveira et al., 2023). All *A. tbilisiensis* isolates were resistant to penicillin G, 44% of the isolates were resistant to ampicillin, while 22% and 11% tested as resistant to ciprofloxacin and gentamicin, respectively, which is in agreement with other studies (Chaiyasaen al. 2023; Rahimi, 2014). Resistance to gentamicin is quite rare, although there are a few studies that have reported it. None of the *Arcobacter* isolates were resistant to erythromycin, contrary to studies conducted in Europe and Asia that have reported erythromycin-resistant isolates of *Arcobacter* spp. (Oliveira et al., 2023). None of *A. tbilisiensis* sp. nov. isolates appear to be MDR strains.

The antibiotic susceptibility profiles of *C. jejuni* and *C. coli* were somewhat different. All isolates of both species were resistant to penicillin G. Resistance to ciprofloxacin was high

among both *C. jejuni* and *C. coli* isolates (79% and 97%, respectively). None of the *C. jejuni or C. coli* isolates were resistant to erythromycin, while 28% and 51%, respectively, demonstrated resistance to ampicillin. Resistance of *C. jejuni* and *C. coli* to tetracycline was also high (28% and 51%), which in fact is quite common and agrees with other studies. For example, a recent study by Poudel et. al found that 23.7% of *C. jejuni* isolates that came from chickens raised at NAE (no antibiotic ever) farms in the state of Mississippi were resistant to tetracycline(Poudel et al. 2022).

A few isolates of both *C. jejuni* and *C. coli* demonstrated resistance to streptomycin, while a single isolate (3%) of *C. coli* tested resistant to chloramphenicol. 19 *C. jejuni* isolates showed resistance to three different groups of antibiotics (penicillins, fluoroquinolones and aminoglycosides) and one isolate (CJ 9) was identified as an MDR strain, as it was additionally resistant to erythromycin and chloramphenicol.

It is important to note that local practices of antibiotic use in meat production industries of specific geographical locales play the decisive role in the antibiotic resistance profiles among the local isolates of *Arcobacter* spp. and *Campylobacter* spp. For example, while many studies report resistance to ampicillin among *Arcobacter* strains isolated from chicken meat, the isolates obtained by Jribi et al. were not resistant to this drug (Jribi et al., 2020). A Turkish study identified some cases of resistance to chloramphenicol (3%) and streptomycin (1%) among the chicken isolates of *A. cryaerophilus* (Yesilmen et al., 2022), which we observed among *A. tbilisiensis* sp. nov. isolates too.

Based on the findings of multiple research groups, it is evident that resistance to ciprofloxacin is going to continue to rise in *Arcobacter* spp. due to widespread antibiotic use in local human and veterinary medicine.

7.6. Survival of *Arcobacter tbilisiensis* sp. nov. in river water under refrigeration

Many studies have reported the special relationship of *Arcobacter* spp. with water and that water is the main factor in transmission of *Arcobacter* spp. (Šilha et al., 2021; Lee et al.,

2012). Data on water survival of the *Arcobacter* isolates in the present study are close to those of Moreno et al. who determined the survival time of *A. butzleri* NCTC 12481 in non-chlorinated drinking water to be 16 days (Moreno et al., 2004). Most *A. tbilisiensis* sp. nov. isolates survived and could be cultured after 10 days under refrigeration in the water obtained from the River Elbe, while only one isolate could be cultured on day 14. Previously Pérez-Cataluña et al. established the fact that temperature played a significant role in the length of survival of *Arcobacter* spp. in the environmental waters and sewage. For example, temperature played a determinant role in which *A. cryaerophilus* type could be isolated-1A or 1B-from sewage waters (Pérez-Cataluña et al., 2018).

An important finding is that, as observed with Campylobacter spp., *Arcobacter* spp. are also able to survive inside the crevices on chicken skin and inside the chicken carcass, thus potentially serving as a source of infection.

7.7. Cytotoxic effect of *Arcobacter* spp. on CaCo-2 cells

The WST1 assay, which we used to determine the cytotoxic effect of the *Arcobacter* isolates on CaCo-2 cells is based on the ability of mitochondrial dehydrogenases produced by *live cells* to cleave WST1 transforming it into formazan. This is a quantitative colorimetric reaction and, after reaching the endpoint, it may be "read" on a spectrometer. We found that the cytotoxic effect of *A. tbilisiensis* isolates ranged between 52-87%, meaning that after exposure to *Arcobacter* spp., 52 to 87% cells were damaged, compared to the negative control. The NC was not exposed to *Arcobacter* spp., thus its OD reading corresponded to 100%. After a simple calculation, the cytotoxicity effect could be determined from the OD readings. First, we were surprised by the results, because previously Brückner et al. reported lower cytotoxicity (10%) of *A. cryaerophilus* isolates using HT-29/B6 cells in WST1 assay (Brückner et al., 2020). Generally, *A. butzleri* demonstrates higher cytotoxicity than *A. cryaerophilus* (Brückner et al., 2020). Compared to *A. cryaerophilus, A. tbilisiensis* sp. nov., also appears to be more virulent due to the presence of many *Campylobacter*– like virulence factors that are discussed below.

7.8. Virulence factors identified in *A. tbilisiensis* sp. nov.

The genera *Arcobacter* and *Campylobacter* are closely related and share many virulence factors, such as CiaB, which we identified in all *A. tbilisiensis* sp. nov. isolates. Douidah et al. examined different reference species of *Arcobacter*, such as *A. cryaerophilus*, *A. butzleri* and *A. skirowii* isolated from animals and humans, for the presence of the known virulence factors. The c*iaB* gene was present in all the reference strains. It has been suggested that in *C. jejuni* ciaB is involved in promoting internalization of this pathogen during host invasion- a complex process that requires different factors, e.g. a full-length flagellar filament, among others (Fanelli et al. 2019). Douidah et al. determined that out of 34 *A. cryaerophilus* strains isolated from the Belgian chicken meats, 33 contained the *ciaB* gene. At the same time, the *cadF* gene coding for a calcium-dependent *Campylobacter* adhesion factor was present only in 5 out of total 34 (15%) chicken isolates. In contrast, all 36 *A. butzleri* strains isolated from chicken meat carried this gene (100%) (Douidah et al. 2012). Interestingly, all *A. tbilisiensis* sp. nov. isolates in this study also carry the *cadF* gene. Additionally, similarly to the Belgian isolates of *A. butzleri*, *A. tbilisiensis* sp. nov. isolates carry other virulence genes at higher frequencies (Table 3) compared to *A. cryaerophilus* isolates cultured from chicken meat. This supports the experimental evidence from the cytotoxicity study conducted in this study suggesting that *A. tbilisiensis* sp. nov. may be as virulent as *A. butzleri.* On the other hand, we did not see *hecA* and *hecB* hemaglutinins in any of the *A. tbilisiensis* sp. nov. strains, possibly be due to the fact that these virulence factors have been poorly characterized in *Arcobacter* spp*.* and the NCBI database does not contain complete annotated sequences of these genes. Interestingly, the *pldA* gene, coding for a phospholipase, was present only in 3% of the chicken isolates of *A. cryaerophilus*, whereas 100% of *A. butzleri* isolates carried it (Douidah et al. 2012). Using PCR, Sekhar et al. identified all virulence genes, also seen in *A. tbilisiensis* isolates of this study (Table 3), among the *A. butzleri* and *A. cryaerophilus* strains of human and animal origin. However, while all *A. butzleri* isolates carried the *pldA* gene, few of the *A. cryaerophilus* isolates contained them, which pinpoints c*adF* and *pldA* as very

important virulence determinants in *Arcobacter* species (Sekhar et al. 2017). Of note, all of *A. tbilisiensis* sp. nov. isolates also carry the *pldA* gene.

Our findings are comparable to those of a 2021 Estonian study, which identified *ciaB* and *mviN* in all *Arcobacter* strains consisting of *A. cryaerophilus* and *A. butzleri* isolated from human, environmental and food samples. In this study *cj1349* and *tlyA* were detected only in a few *A. cryaerophilus* isolates. Additionally, none of the *A. cryaerophilus* and all of the *A. butzleri* strains carried the *cadF* gene. The rest of the genes, such as *irgA*, *iroE*, *hecA* and *hecB* were infrequently present in the *A. butzleri* isolates (Uljanovas et al., 2021). Again, the fact that *cadF* was present in all of *A. butzleri* isolates underlines the importance of this gene as a virulence determinant. Alternatively, since *ciaB* and *mviN* genes were found in all *A. cryaerophilus* isolates in a 2020 German study, it is possible that these genes are associated with less virulence and were responsible for the low cytotoxicity of the strains used by Brückner et al. (Brückner et al., 2020). The importance of *cadF* was also confirmed by a Turkish study, which identified this gene in all (100%) of *A. butzleri* isolates, while only 55% of *A. cryaerophilus* isolates carried it. A significant number of the *A. cryaerophilus* strains in the same study contained *ciaB* and *mviN* (Tabatabaei et al., 2014).

Due to the use of different detection methods (e.g. PCR vs NGS), the presence of various virulence factors in *Arcobacter* spp. may differ from study to study. The high frequency of similarities between various genomic regions makes designing specific primers essential for the detection of virulence genes. Incorrectly designed primers could lead to false positive results. However, false negative results are also possible due to DNA quality and other technical challenges.

Proteins involved in the process of orchestration of chemotaxis, motility, and signal transduction are crucial to the survival of microorganisms. These proteins/factors determine the ability of microbes to colonize different ecological niches. Thus all these proteins, including the adherence factors, which help a microbe to affix to different kinds-often even abiotic-surfaces are frequently involved in pathogenesis and antibiotic resistance. From this

point of view, various proteins involved in iron metabolism and resistance to heavy metals, are also qualified as virulence factors. Regions coding for such genes were found in all *A. tbilisiensis* sp. nov. isolates. For example, RAST identified several mycobacterial virulence operons possibly involved in quinolinate and protein syntheses, as well as DNA transcription. Other virulence factors identified in *A. tbilisiensis* sp. nov. isolates were the genes coding for proteins involved in flagellar rotation and movement (*flgB*, *flgC* and *fliE*), iron metabolism and oxidative stress (e.g. ferric iron transporter and ferric ion binding proteins and proteins involved in ferric uptake and peroxide stress regulation), which *Arcobacter* spp. and *Campylobacter* spp. have in common.

A. tbilisiensis sp. nov. isolates discussed in this dissertation do not contain pVIR–a plasmid previously identified both in *Campylobacter* spp. and *A. butzleri* (Zautner et al., 2023).

7.9. Identification of antimicrobial resistance genes in *A. tbilisiensis* sp. nov. isolates

RAST (Rapid Annotation using Subsystems Technology, https://rast.nmpdr.org/) and the NCBI's Prokaryotic Genome Annotation Pipeline (PGAP, https://www.ncbi.nlm.nih.gov/genome/annotation_prok/) were used to identify antibiotic resistance genes in *A. tbilisiensis* sp. nov. isolates. PGAP, based on protein homology, suggested protein sequences for various resistance genes, which were reverse-translated, blasted to the reference genomes of *Arcobacter* spp. and consequently mapped back to the *A. tbilisiensis* sp. nov. genomes.

Before delving into the specific resistance genes identified in *A. tbilisiensis* sp. nov. isolates, the mechanisms of resistance need to be mentioned. Among the different resistance mechanisms that exist, enzymatic inactivation of the drug, active efflux of the antibiotic drug and the drug target site mutations are the mechanisms that we detected in the *A. tbilisiensis* sp. nov. isolates.

Among all isolates of *A. tbilisiensis* sp. nov., type D beta-lactamase and SMR (Small Multidrug Resistance) protein were detected both by RAST and PGAP. For example, blaOXA, a class D beta-lactamase found in isolate 51 (QT384_02110: 399612..400373)

mapped to the genomes of *A. tbilisiensis* sp. nov. isolates with various degrees of percent coverage and percent identity after having been reverse-translated and blasted to the reference *Arcobacter* genome (OM617734.1). More than 1,000 different β-lactamases which, of note, are capable of destroying 3rd generation cephalosporins, have been identified to date (www.lahey.org/studies) (Munita and Arias, 2016). The presence of related class D β-lactamases was identified in *Arcobacter* spp by Iranian and French researchers as well. They found blaOXA-61 and blaOXA-15/464-like family proteins, respectively, in *A. cryaerophilus* and *A. butzleri* isolates originating from milk samples and the genomes of 30 resistant *A. butzleri* isolates that were purposefully analyzed for determining cutoff values for various antibiotics, including ampicillin (Jehanne et al. 2022; Lameei et al. 2022). Evidently, beta-lactamase family incorporates a large number of related proteins. Not all *A. tbilisiensis* sp. nov. isolates, however, displayed resistance to ampicillin phenotypically. From the strains that did show resistance to ampicillin, isolate 46's genome almost fully mapped to OM617734.1 while isolate 47's genome, mapped to the latter only partially (23%). This indicates that resistance to ampicillin does not depend on solely the presence of blaOXA family of genes, especially when the genomes of the isolates 79 and 99 were not resistant to ampicillin despite high similarity with the blasted reference gene.

Another enzyme identified in the *A. cryaerophilus* genomes by PGAP was CatB-related O-acetyltransferase/Chloramphenicol Acetyltransferase referenced in the *A. tbilisiensis* sp. nov. isolates 51 (QT384_06745: 1311915..1312487) and 46 (QUR77_02155: 413946..415091) although without the phenotypically demonstrated resistance to chloramphenicol. Blasting the protein sequence using NCBI's delta blast function yielded various hits, such as catB-Related O-acetyltransferase of *Alphaproteobacteria* bacterium (GenBank: MBQ7413357.1) with 84% coverage and 46% identity, as well as a related gene in *Clostridium* spp. (Sequence ID: CDC18198.1) with 84% coverage and 51% identity. However, after reverse-translating the protein sequence and blasting it using the NCBI's nucleotide blast function we identified hits that were closer to the isolate 51: *A. cryaerophilus* D2610 (GenBank CP032825.1) with

53% coverage and 74% identity. We then blasted CP032825.1 back to our isolates one by one and found that this gene was present in all *A. tbilisiensis* isolates.

The presence of the *catB* gene-also a Chloramphenicol Acetyltransferase-was previously reported by Fanelli and colleagues. According to their research, *A. butzleri* LMG10828 demonstrated "intermediate" resistance to chloramphenicol (Fanelli et al. 2019). However, resistance of *Arcobacter* spp. to various antibiotics has not been clearly defined to date. This is also true for resistance of *Arcobacter* spp. to chloramphenicol, which involves enzymatic inactivation of the drug by mostly acetyltransferases. Additionally, the presence of efflux pumps, phosphotransferase enzymes coupled with other drug resistance mechanisms, such as modifications of the drug target site via point mutations and decreased outer membrane permeability, may also contribute to chloramphenicol resistance.

Another enzyme identified by PGAP pipeline in isolate 51 was Cephalosporin Hydroxylase family protein (QT384_06750: 1312526..1313314), however, even though blasting the given protein sequence using delta blast did identify Cephalosporin Hydroxylase in various bacteria, reverse-translating the protein sequence followed by nucleotide blast did not yield any relevant sequence associated with this enzyme in any of *A. tbilisiensis* sp. nov. isolates.

The mechanism of active efflux of antibiotic drugs is a major player in antibiotic resistance. Over-expression of drug efflux pumps is an important and sufficiently well-studied multidrug resistance mechanism in Gram-negative bacteria. These efflux systems have evolved to export various antibiotics from cytosol keeping their concentration below what is sufficient to act on corresponding cellular targets. Additionally, efflux pumps serve as channels extruding molecule implicated in bacterial toxicity, quorum sensing and biofilm formation thus contributing to the pathogenicity of gram-negative bacterial species (Webber et al. 2022). Resistance Nodulation Division (RND) and ABC (ATP-binding cassette) transporter families, identified in all *A. tbilisiensis* sp. nov. strains, are often found in Gram-negative bacteria and are clinically relevant due to the ability to effectively remove a wide range of antibiotics from the bacterial cell (Nishino et al., 2021; Vargiu et al. 2022). Cryogenic

electron tomography (cryo-ET) of whole bacterial cells revealed that RND and ABC-type efflux systems consist of tripartite assemblies, which include an inner membrane component, an adaptor membrane fusion protein (MFP)/periplasmic adaptor protein (PAP) of the periplasm, and an outer membrane factor (OMF). The PAP is serving as the connector between the RND transporter and OMF. RND family transporters are engaged in the catalysis of active efflux of many chemotherapeutic drugs, dyes and a wide range of antibiotics thus contributing to bacterial multidrug resistance (MDR) and making development of new antibiotic compounds extremely challenging (Webber et al. 2022; Nishino et al., 2021). RND pumps are usually encoded by chromosomal DNA, however one worrying example is that such system was found to be carried on an IncH1 plasmid of *Citrobacter freundii* strain along with the gene NDM1 coding for the enzyme New Delhi metallo-β-lactamase 1(Blair et al. 2015).

Resistance to erythromycin among *Arcobacter* spp. has been reported by several research groups. For example, Sciortino et al. found that 7.4% of *A. butzleri* strains isolated from various water sources in Italy were resistant to this drug (Sciortino et al. 2021). Van Den Abeele and colleagues, on the other hand, determined that 19% of the *Arcobacter* spp. used in their study were resistant to erythromycin. Such high rate of resistance observed in this particular study was probably due to human origin of the isolates and the use of gradient diffusion method in susceptibility testing, as opposed to more frequently used disc diffusion method (Van Den Abeele et al. 2016). Resistance to macrolide drugs mainly occurs due to point mutations within domain V of the 23S rRNA gene and/or amino-acid substitutions in the ribosomal proteins (Uljanovas et al. 2023). Such mutations in the 23 rRNA were identified by Chinese researchers investigating *Campylobacter* spp. in central China with many isolates were shown to be resistant to erythromycin (Cheng et al. 2020). Another mechanism of resistance relies on active efflux of the drug using the macABC efflux pumps.
The macAB efflux system consisting of macB type I secretion system permease/ATPase (QT384_10165: 1991339..1993504), MacC HlyD family type I secretion periplasmic adaptor subunit (QT384_1017: 1993501..1994919) and a tolC Type I secretion outer membrane

protein (QT384_10160: 1988747..1991320) that represents ABC superfamily was identified in all *A. tbilisiensis* sp. nov. isolates by blasting the reverse-translated protein sequences suggested by PGAP. Pérez-Cataluña et al. identified MacAB-TolC system in all 52 isolates of *A. cryaerophilus* originating from a wide-range of samples sourced from various countries (Pérez-Cataluña, Collado, et al. 2018). MacAB has been implicated in macrolide and aminoglycoside resistance in *E. coli* and *S. enterica* serovar *typhimurium* as well (Shirshikova et al. 2021).

Despite the presence of the macAB efflux system, none of the *A. tbilisiensis* sp. nov. isolates were resistant to erythromycin, which may indicates that, at least in vitro, its presence alone is insufficient for resistance to macrolides. A similar circumstance was observed in the study of 40 Lithuanian *A. butzleri* isolates conducted by Uljanovas et al. in 2020: while all isolates contained the macAB efflux system, not all (55 %) were resistant to macrolides. It was suggested that either the genes were not expressed, or their coding sequence could contain amino acid substitutions (Uljanovas et al. 2023). Similarly, DNA gyrase subunit type II resistant to fluoroquinolones was identified in all *A. tbilisiensis* sp. nov. sequences, however only 4 out of 18 sequences were phenotypically resistant to ciprofloxacin, which implies that this gene acts in conjunction with other mechanisms/genes.

Resistance to quinolones and bile acids in bacterial species may be conferred by cmeABC efflux pumps. The sequence of proteins that make up these tripartite channels were suggested by PGAP. The sequences were reverse-translated to search for similar sequences using NCBI's nucleotide blast. The closest match was then selected and blasted to the genomic sequences of the *A. tbilisiensis* sp. nov. isolates. Thus, cmeB: (QT384_09555 1865926..1869078) multidrug efflux RND transporter permease subunit, inner membrane proton/drug antiporter (RND type), cmeA (QT384_09560 1869081..1870118) efflux RND transporter periplasmic adaptor subunit, membrane fusion component and cmeC (QT384_09565 1870115..1871347) TolC family protein, outer membrane factor lipoprotein, were identified in all isolates of *A. tbilisiensis* sp. nov., after having been blasted to various *Arcobacter* spp. (Table 9). Perez-Cataluna et al. detected cmeABC multidrug efflux pump,

macAB-tolC system for macrolide resistance and the oxqB for quinolone resistance in all sequenced *Arcobacter* genomes in their research (Pérez-Cataluña et al., 2018).

Table 9. Antibiotic resistance genes mapped to the *A. tbilisiensis* genomes

Query ID	Organism	Gene	Isolate	Query Coverage (%)	Identity (%)
CP032825.1	*A. cryaerophilus* D2610	catB-related o-acetyltransferase	All isolates	100	97.4-97.86
CP053839.1	*A. lanthieri* LMG 28516	cmeB	All isolates	100	82
CP031367.1	*A. trophiarum* LMG 25534	cmeA efflux RND transporter periplasmic adaptor subunit	All isolates	100	86
CP060264.1	*A. cryaerophilus* strain 16CS0369-1-AR-4	cmeC, tolC family protein, outer membrane factor lipoprotein	All isolates	100	97
CP053839.1	*A. Lanthieri* LMG 28516	macB type I secretion system permease/ATPase	All isolates	100	85 -88
LT906455.1	*A. butzleri* strain NCTC12481	tolC family outer membrane protein	All isolates	70-72	81-82
CP034309.1	*A. skirrowii* strain A2S6	macC HlyD family type I secretion periplasmic adaptor subunit	All isolates	77-81	86
OM617734.1	*A. butzleri* strain 2015-0489	blaOXA	46, 51, 62, 65, 70, 79, 99	98	75.8
			47,48,49,53, 101,103, 107,108	21-23	95. 8

ABC transporters other than macABC were also present in *A. tbilisiensis* sp. nov. isolates. Notably, metal (Zn, Fe^{2+} and Mb) and peptide ABC transporters, all of which are associated with virulence due to the ability to actively expunge various drugs outside the bacterial cell by using the free energy obtained from ATP hydrolysis and thus facilitating the transport of various substrates across the lipid membrane (Nishino et al., 2021). SMR (Small Multidrug Resistance) protein-another group of proteins that belong to SMR family of efflux pumps was also identified in all *A. tbilisiensis* sp. nov. isolates. These efflux systems are composed of small proteins with four very hydrophobic transmembrane domains that have α-helical structure (Bay, Rommens, and Turner, 2008). SMRs are integral inner membrane proteins of around 12 kDa in size and range from 100 to 140 amino acids in length. The SMR protein family members have been found on various plasmids and transposable elements and provide resistance to a wide range of antibiotics, such as β-lactams, cephalosporins and aminoglycosides. SMR proteins frequently occur in combination with other drug resistance genes indicating a tight association between antibiotic and SMR resistance (Bay, Rommens, and Turner, 2008).

Several mechanisms regulate resistance to tetracycline in bacterial cells: efflux, modification and protection from binding to the ribosome and modification of 16S rRNA at the tetracycline binding site. Various proteins regulate these mechanisms: for example Tet(O) and Tet(M), which are translational GTPase EF (Elongation Factor)-G paralogs able to remove tetracycline from the inhibitory site on the ribosome via a GTP-dependent hydrolysis. Tet(O) and Tet(M) belong to ribosomal protection proteins (RPPs) along with other proteins of similar function, such as Tet(Q), Tet(S), Tet(T), Tet(W), and OtrA (Fanelli et al., 2019). Despite the presence of the Translation Elongation Factor G in all *A. tbilisiensis* sp. nov. strains, none were resistant to this antibiotic. At the same time, many strains resistant to tetracycline were seen among the *Campylobacter* spp. isolated from the same material.

7.10. Effect of *L. fermentum* on *Campylobacter* spp. and *Arcobacter* spp.

Having demonstrated the inhibitory effect of *L. fermentum* on *Campylobacter* spp. we searched for available publications involving this bacterium. *L. fermentum* turned out to be a known probiotic that has been used in conjunction with Zn (II) propionate to significantly decrease *Salmonella enterica* serovar *Düsseldorf* fecal shedding in mice (Mudroňová et al., 2006). The combination of *L. fermentum* and Zn also promoted weight gain in chicks, compared to the NC group. Another study conducted in a mouse model, achieved eradication of *S. typhimurium* using *L. fermentum* ME-3 in combination with ofloxacin. This treatment eliminated *S. typhimurium* in the animals' blood, ileum and liver, while decreasing the number of animals with liver and spleen granulomas. The absence of liver granulomas was associated with higher counts of intestinal lactobacilli in all experimental groups (Truusalu et al., 2008).

There are numerous publications providing many examples of inhibition of various pathogenic bacteria by different *Lactobacilli*, but oftentimes with results that either contradict each other, or can not be compared to each other due to the use of different assays, media and/or experimental conditions. In a solid study the process of characterization and selection for a LAB strain able to inhibit a certain pathogen must be based on a validated assay with clearly defined parameters, while each assay run must be evaluated for consistency of the results. Only in this manner one can determine the mechanism by which a given probiotic inhibits a certain pathogen.

8 Conclusions, outlook and recommendations

Based on the presented research, it can be concluded that Georgian retail chicken is contaminated with *Arcobacter* spp. and *Campylobacter* spp. and a great way to move forward from here would be to determine the frequency of these pathogens in meat of other animals, for example pork and beef. Additionally, we think that *A. butzleri* and *A. cryaerophilus* can also be found in local retail chicken meats. This would make another

excellent research project, as well as investigating the prevalence of *Arcobacter* spp. and *Campylobacter* spp. in the environmental waters and other types of meat.

Based on our findings the following conclusions can be made:

1. Prevalence of *Campylobacter* spp. in Georgian retail chicken meat could be ranging from 50% to 70% or more, based on the ratio of the number of isolates (n=92) and the total number of samples screened (n=200). We assume that the actual numbers are much higher than what we have obtained.
2. The ratio of *C. jejuni* and *C. coli* among the isolates is roughly 50%:50%. However, additional studies are needed to confirm this finding. This ratio could vary in other parts of the country, or from farm to farm.
3. The presence of *Arcobacter tbilisiensis* sp. nov. in Georgian chicken is not surprising, however, we do not know whether the ratio of the number of isolates (n=19) and the samples screened (n=200) is true. Because the isolates did not include either *A. butzleri* or *A. cryaerophilus,* we think that this ratio is in reality much higher.
4. According to the susceptibility resistance testing, the three major groups of antimicrobials that the *Campylobacter* isolates are resistant to are beta-lactams, cephalosporines (ciprofloxacine) and tetracyclines. All *C. jejuni* and *C. coli* isolates (n=74) were resistant to penicillin G, 79% of *C. jejuni* and 97% of *C. coli* were resistant to ciprofloxacine while 28% of *C. jejuni* and 51% of *C. coli* were found to be resistant to tetracycline.
5. All isolates of *A. tbilisiensis* sp. nov. were resistant to penicillin G. and 22% of the isolates were resistant to ciprofloxacin.
6. *L. fermentum* is able to inhibit all three pathogens in vitro overnight in the co-incubation assay, while such inhibition was not observed in well diffusion and disk diffusion assays, which indicates that cell to cell contact is needed to trigger such inhibition.

7. Cytotoxicity of the local isolates of *C. jejuni* and *C. coli.* and *A. tbilisiensis* sp. nov., based on Roche's WST1 cell proliferation assay, ranges from 52% to 87%, which means that the isolates exhibit significant virulence.
8. The same WST1-based cytotoxicity assay demonstrated that *L. fermentum* protects CaCo-2 (human colon carcinoma cell line) cells from cytotoxic effect of local isolates of *C. jejuni*, *C. coli* and *A. tbilisiensis* sp. nov., completely eliminating cytotoxicity.

The most important finding of this research has been that the locally produced chicken meat is contaminated with *Campylobacter* spp. and *A. tbilisiensis* sp. nov. This requires meat producers to address the contamination issue. Based on our findings, the following measures are recommended to decrease *Campylobacter* spp. and *Arcobacter* spp. in raw chicken meat:

1. **Improving** the sanitation and biosecurity of the farm facilities, such as hatcheries and abattoirs
2. **Sampling** of worker boots periodically and performing qPCR testing for *Campylobacter* spp. should provide information on the prevalence of these pathogens in farms
3. **Water** facilitates transmission of *Campylobacter* spp., therefore clean drinking water is absolutely necessary for the poultry.
4. **Testing** flocks for *Campylobacter* spp. at 2-3 weeks of age and separation of the flocks based on the positivity and the negativity of the Campylobacter testing.
5. Provide a **balanced diet** for birds.
6. Administer a probiotic formula to the chicks via food and/or water. This would require prior testing of the efficacy of such formula. This can be done at the age of three weeks and then a few days before killing, so that the *Campylobacter* load in the chicken guts diminishes.
7. Utilize available *Campylobacter* phages isolated locally by administering them in drinking water for birds.

8. Any equipment, production line, surfaces that come in contact with chicken carcasses must be properly cleaned and disinfected prior and after handling raw chicken meat.
9. qPCR testing of carcasses for *Campylobacter* to sort the meats into campy-positive and campy-negative carcasses. Freezing positive carcasses will eliminate *Campylobacter* spp.

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