

NeO.GiANT

Legacy Document



Funded by
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Our Consortium in Europe



Our Consortium in Latin America



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 NeoGiANT H2020 project



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Our Statement

NeoGiANT has been more than a project; it has been a journey. Over four and a half years, we explored the power of nature through the antimicrobial and antioxidant potential of Albariño grape marc, aiming to improve animal welfare, reduce antimicrobial resistance, and give new life to agri-food waste. This journey brought together nearly 200 people from 20 partners across 9 countries: researchers, entrepreneurs, producers, communicators, students, and managers. Each perspective mattered, teaching us that collaboration is not just a method; it is a mindset. Listening, debating, and learning from each other made us stronger and enriched every decision we took.

We faced challenges: scientific, regulatory, logistical, and global events that tested our resilience. Yet, we also shared unforgettable moments, explored cultures, and discovered that innovation thrives when curiosity, passion, and humanity come together.

This document is not only a reflection of what we achieved; it is a foundation for the future. It will guide and inspire ongoing and future research, ensuring that the knowledge, methods, and insights of NeoGiANT continue to benefit science, industry, and society.

In the end, NeoGiANT reminds us that the greatest discoveries are not just in the lab, but in people, their ideas, dedication, and diversity. Together, we have harnessed nature's potential to create solutions for healthier animals, safer food, and a more sustainable future.

The Neogiant Project team

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Project Overview

NeoGiANT is a large-scale and highly ambitious project based on antimicrobial and antioxidant extracts from white grape marc, specifically from the Albariño variety, which became our daily reality for four and a half years (from October 2021 to March 2026) and whose award made us feel as though the world had gone wonderfully mad.

NeoGiANT is an innovative project that offers new solutions based on the known potent natural antimicrobial and antioxidant activities of grape marc extracts, due to their arsenal of phytochemicals, in particular their phenolic compounds content, to produce enhanced feed, treatment products and natural sperm preservatives for livestock and aquaculture. The project aims to provide effective, natural solutions that can complement conventional antibiotics, not only reducing reliance on them but also mitigating the risks of developing AMR in livestock and aquaculture. The extracts contain a complex mix of bioactive polyphenols that act through multiple mechanisms, making it harder for bacteria to develop resistance compared to single-compound antibiotics. In this way, the scope of application of conventional antibiotics—which are, of course, essential—will be reinforced, as their use will be restricted to situations where they are truly necessary.

Being aware that a multidisciplinary team is needed to tackle any complex problem, the project, funded with over €8.3 million, was carried out by a consortium coordinated by USC and comprising 20 partners from nine countries (ES, UK, PL, CZ, HU, DE, PT, BE and AR): 4 higher education institutions (the Universities of Santiago de Compostela, Porto, Berlin and South Bohemia in the Czech Republic), 5 research organisations (MRI, IBPRS, VRI, MATE and

CONICET), 8 companies – 5 SMEs (iGrape, Contactica, Anitom, LifeBioEncapsulation, Bianor Biotech) and 3 large companies (CZ Vaccines, Magapor and Nutrition Science) – as well as the Association of Young Farmers (ASAJA) and the Spanish Association for Standardisation (UNE). The ultimate aim is to reduce the emergence of new antimicrobial resistance (AMR) and better control existing resistance in the field of farming and breeding animals intended for human consumption.

Together, we have learnt so much on this incredible journey – about management, science, technology, regulation, politics, business... It has been such an exciting journey that it is very difficult to sum it up in just a few paragraphs. Not forgetting how much we have learned from the historical and gastronomic heritage of a good number of European countries... But above all, we have confirmed that well-managed collaborative work leads to giant leaps forward and that all opinions are important, absolutely all of them. Another incredible thing about this consortium is the diversity of the partners' professional profiles. This is crucial. As researchers, we have a point of view (which we usually tend to think is the correct one), but as soon as you listen carefully to the people from companies and business, you realise that it may not be exactly how you see it. And when you add consumers and end users and their associations, suddenly another point of view is included that you hadn't even thought of...

This project is not one project, but many. We knew that from the outset. Not only because each area—animal feed, animal health and animal reproduction—is a world in itself, but also because, not satisfied with covering all three areas, we wanted to apply our innovative solutions

to four animal groups: chickens, cows, pigs and fish. What's more, we soon discovered that the only thing a sea bass, a salmon, a turbot and a rainbow trout had in common was that they were all fish, but each animal species we wanted to tackle would be a project in itself.

But then, there were all the other areas: the production of useful extracts in various formats, their scaling, the extrapolation of in vitro results to in vivo, in-depth research into the effects of our polyphenols on the immune system, zootechnical aspects, the eco-design of NeoGiANT products, life cycle analyses, pre-commercial validation, and the scaling of prototypes to pre-industrial scale. But also, regulatory issues, market analyses, exploitation of project results, intellectual property management, legal requirements,... and much more.

Not to mention cooperation with European institutions, the Green Deal support office, other sister projects in the Farm-to-Fork programme, and the need to convert everything we had learned into policy recommendations so that the European Union could receive feedback and take our contributions and turn them into useful management tools. In other words, we tried to be useful in defining new European policies related to our project.

Not forgetting that we are publicly funded, an extremely important detail that drove us, from the beginning, to try to ensure that formulations based on NeoGiANT functional ingredients become a reality within the reach of the society that has funded them, and with the appropriate scientific and technical support developed in the corresponding project work packages. And, of course, we had to let the whole world know about it, tell everyone, through our invaluable

Communication and Dissemination plan, which has taken our achievements very far.

Despite the enormous amount of work, the thousands of meetings, the stressful moments the difficulties, the challenges, the countless emails piled up like cells in perpetual mitosis..... Honestly, NeoGiANT has been both professionally and personally outstanding for all of us. We have learnt an awful lot together and about a wide range of topics; we now have a much better understanding of every single aspect of the project, and we've also gained an 'honours degree' in human relations through our interactions with a diverse group of people from a wide variety of countries.

To conclude, whether it is grape marc or any other source of plant bioactives, in the end, the key is the power of nature... Our only merit is to have explored various ways to harness that power to solve specific problems, improve animal welfare, contribute to reducing antimicrobial resistance and, ultimately, improve human health through the consumption of healthier animals, while helping to reduce agri-food waste through its reuse.

Thank you to everyone who has contributed to making this project truly legendary.

Mission accomplished.



WP2 Grape extract production & natural formulations with antimicrobial properties

1. Scientific & technical achievements

■ What were the main scientific and technical achievements

WP2 achieved extensive scientific and technological progress in the use of natural extracts for veterinary use to manage infectious diseases by establishing an integrated pipeline that transforms grape marc, an abundant agricultural byproduct, into a series of antimicrobial formulations. The work began with the optimization of the extraction processes using GRAS solvents, enabling the production of high-polyphenol extracts with strong antioxidant and antimicrobial activity. These extracts underwent full analytical characterization and were successfully scaled from laboratory conditions to small-scale and later pre-industrial production, demonstrating the reproducibility and robustness of the methods. In parallel, the work package developed a set of solid and liquid formulations tailored to veterinary, aquaculture, and reproductive applications. Spray-dried powders with high phenolic retention were obtained for feed applications, while disease-targeted formulations for mastitis, exudative epidermitis and fish diseases were designed, characterized and optimized. Each prototype was assessed for stability, physicochemical suitability and antimicrobial activity, ultimately delivering a range of promising natural alternatives to conventional antibiotics.

■ How were they addressed?

These achievements were the result of a structured methodological workflow combined with ongoing collaboration with other work packages. Initially, a wide array of extracts was produced and thoroughly evaluated for bioactive content, antioxidant capacity and antimicrobial activity. Based on these results, formulation efforts progressed through iterative cycles of design, testing and refinement. Close cooperation with work packages 3, 4, 5 and 6 ensured that formulations were evaluated not only for their physicochemical properties but also for biological performance, toxicity, antimicrobial efficacy and

suitability for in vivo use. Throughout this process, key parameters such as viscosity, pH, bioadhesion, osmolality and storage stability were carefully monitored, allowing systematic refinement of each prototype. The evaluation of sterilization strategies further supported the robustness of the products, with gamma irradiation selected as the preferred method due to its compatibility with the formulations. This coordinated and evidence-driven approach enabled WP2 to efficiently address technical challenges and deliver high-quality, stable prototypes.

■ What key methodologies or technologies were developed?

WP2 generated multiple technological innovations. The extraction processes resulted in optimized green methodologies that use food-grade solvents and statistically guided operating conditions, ultimately evolving into a reproducible pre-industrial process. Formulation development introduced sophisticated delivery systems such as mucoadhesive polymer dispersions, contributing to enhanced residence time and controlled release in topical and intramammary applications. Additional innovations included controlled-release polymer matrices for aquaculture, and efficient spray-drying processes for creating stable, functional powders with minimal excipients. These were supported by validated sterilization methods, detailed Standard Operating Procedures for production at semi-technical scale, and comprehensive analytical workflows for monitoring polyphenol content, viscosity and long-term stability.

2. Key Environmental Impact

■ What are the most significant contributions of your WP to NeoGiANT?

WP2 contributed decisively to NeoGiANT by developing the full suite of grape-marc extracts, powders, and antimicrobial formulations required to achieve the project's scientific and technological objectives. Through optimized

extraction procedures, green solvent selection, and successful scale-up to pre-industrial production, the work package transformed an agricultural residue into high-value, bioactive materials. These materials were essential for enabling the project's investigations into alternatives to antibiotics across multiple animal health applications.

A second major contribution of WP2 was the creation of robust, application-specific formulations including intramammary preparations, topical sprays, liquid systems for aquaculture, liquid and solid feed ingredients, and semen extender-compatible prototypes. Each formulation was rigorously characterized in terms of stability, physicochemical properties, sterilization compatibility and bioactive retention. This ensured that all prototypes entering subsequent project stages were reliable, standardized, and suitable for safety, efficacy, physiological and pre-commercial testing.

Finally, WP2 played a central role in NeoGiANT. By supplying consistent, well-characterized materials and standard operating procedures, the work package ensured comparability across studies, facilitated process optimization at larger scales, and provided the basis for sustainability, regulatory and exploitation assessments. In parallel, its researchers actively contributed to communication and dissemination activities, helping to highlight the project's advances in natural antimicrobials and sustainable animal health strategies.

■ How can these results be used in future projects or policies?

The results of WP2 provide a strong technological base for future research and innovation actions focused on natural antimicrobials, sustainable livestock production, and circular valorization of agri-food by-products. The optimized extraction processes, scalable formulation methodologies, and validated prototypes can be directly transferred to new projects aiming to reduce antibiotic use in animal health or to develop bioactive ingredients from agricultural waste.

Because these processes have already been tested from laboratory to pre-industrial scale, they offer a mature starting point for industrial pilots, regulatory testing, and further Horizon Europe initiatives. A major policy-relevant outcome is the publication of CWA 18342:2026, authored by WP2 researchers and released through CEN. This document provides harmonized guidelines for antioxidant assessment in extracts from white grape marc and establishes a pre-standardization reference that can be adopted by industry, laboratories, and regulators. The CWA strengthens the potential for future European Standards (EN) and supports the creation of certification schemes and regulatory frameworks for natural bioactive products. It also facilitates comparability, quality assurance, and market acceptance across future research or commercial initiatives involving grape-marc extracts or similar agri-food by-products. These results can inform evidence-based policies on antimicrobial resistance reduction, sustainable aquaculture, and circular bioeconomy strategies. The demonstrated feasibility of converting grape marc into effective antimicrobial and antioxidant ingredients directly supports EU Green Deal and Farm-to-Fork objectives. The methodologies and SOPs developed in WP2 can guide future regulations promoting natural veterinary products, eco-designed formulations, and the valorization of agricultural residues, thereby contributing to more sustainable and resilient food and animal-health systems across Europe.

3. Challenges and Lessons Learned

■ Were there any unforeseen factors that impacted the research?

Several unforeseen factors influenced the activities within WP2. Administrative delays in personnel recruitment temporarily reduced research capacity, requiring internal redistribution of effort. From a scientific perspective, certain challenges emerged during prototype development. In aquaculture applications, some extracts induced toxicity in zebrafish due to osmolality and pH imbalances, requiring significant reformulation work. In veterinary

WP2 Grape extract production & natural formulations with antimicrobial properties

applications, high concentrations of excipients in intramammary prototypes triggered inflammatory responses during preliminary testing, necessitating the redesign of the prototype. In the context of reproductive technologies, sperm sensitivity varied substantially across species, making it more difficult to identify universally compatible extender formulations. Each of these issues required careful evaluation and adaptation of project plans.

■ How did these challenges influence project outcomes?

Although these challenges required additional effort, they ultimately strengthened the quality and relevance of the resulting formulations. The toxicity observed in aquaculture led to the design of controlled-release polymeric matrices that may provide improved environmental performance and reduce risk during administration. The inflammatory response associated with intramammary formulations guided the development of a more refined prototype with lower polymer content and enhanced antimicrobial efficacy. Recruitment issues were overcome as project staffing stabilized, and all planned tasks were completed successfully. The iterative adjustments validated the need for flexible design strategies and highlighted the importance of close collaboration across WPs, ensuring that only the safest and most effective prototypes progressed to in vivo evaluation.

4. Opportunities / Suggestions

■ What potential opportunities emerged from the project that could be further explored?

Multiple opportunities emerged from the scientific advances achieved in WP2. The successful development of natural antimicrobial prototypes opens the door to industrial-scale production of veterinary therapeutics that can replace or complement antibiotics. The strong bioadhesive properties of several formulations suggest potential applications beyond the targeted species and diseases, possibly extending into human dermatology or

wound-care. In aquaculture, controlled-release formulations offer opportunities for environmentally sustainable disease control across multiple species. The spray-dried powders produced at semi-technical scale create further opportunities for developing functional feed additives with antioxidant or immunomodulatory roles. Similarly, the promising results in semen extender compatibility open new avenues for research in reproductive biotechnology.

■ Are there recommendations for future research, industry applications, or policy developments?

Future research should focus on long-term in vivo safety and efficacy studies, dose optimization, and the potential evaluation of extracts combinations to enhance antimicrobial activity. From a policy perspective, the results underline the need for clearer regulatory pathways for natural antimicrobials and incentives that promote the valorization of agricultural byproducts. Encouraging the adoption of sustainable extraction technologies and natural veterinary products could further amplify the environmental and societal impact of the NeoGiANT approach.

5. Please provide three short bullet-point highlights summarising the most relevant or impressive results achieved in your Work Package

- WP2 developed and scaled multiple highly bioactive grape extracts and disease-specific natural antimicrobial formulations across veterinary, aquaculture and reproductive applications.
- Polymeric dispersions using the optimized extracts demonstrated strong stability and in vivo antimicrobial activity in exudative epidermitis.
- Supplementary liquid bioactive ingredients and spray-dried polyphenolic powders were successfully produced at pre-industrial scale with high bioactive retention and excellent stability, demonstrating the feasibility of large-scale grape marc valorization into functional feed ingredients.

WP3 Assessment of the antimicrobial activity in-vitro

1. The main scientific achievement of WP3 was the generation of a comprehensive database compiling the antimicrobial activities (MIC and IC₅₀) of the entire collection of extracts and formulations developed within WP2, evaluated against a wide range of bacteria and parasites responsible for various diseases affecting farm animals and aquaculture species. These antimicrobial activity values were determined in accordance with EUCAST guidelines, using the AlamarBlue assay to quantify cell viability by fluorimetric readout. However, since EUCAST guidelines are primarily designed for assessing the efficacy of antibiotics with well-defined compositions, and given the high chemical complexity of the NeoGiANT extracts, it was necessary to introduce minor methodological adaptations, always in line with EUCAST recommendations, in order to tailor the procedure to the specific characteristics of the materials under study.

2. The results generated within WP3 served as the starting point for the design of the formulations that were subsequently used in the in vivo trials. The data obtained showed that the relationship between the quantitative and qualitative polyphenolic composition of the extracts and their antimicrobial effect exhibited a high degree of variability among the different pathogenic species studied. The extensive experience gained during the project now enables us to rapidly generate large amounts of data on the antimicrobial activity of complex extracts, thereby allowing us to readily undertake the analysis of both new extracts and additional pathogenic species.

3. One of the main challenges arose at the beginning of the project, when we attempted to strictly apply the EUCAST standards for the determination of antimicrobial activity, as the complex chemical nature of the extracts required a different experimental approach. This initially affected the pace of the work, slowing down progress while all groups involved in WP3 aligned

on a common methodology and verified the reproducibility of the results across the different laboratories. Once this initial phase was successfully overcome, results were obtained as expected.

4. One of the opportunities for improvement identified during the project, which would be worthwhile to further explore, is the modification or extension of EUCAST-recommended protocols to better account for the complex chemical composition of natural extracts.

5. - The antimicrobial activity of NeoGiANT extracts is associated not only with their total polyphenol content, but also with the chemical composition of the extracts, which in turn depends on the solvent used for their extraction.

- Due to the complexity of the extracts and the multiple mechanisms of action exhibited by the different molecules they contain, it is difficult to predict their antimicrobial activity against a given microbial species. This highlights the importance of performing in vitro assays prior to the development of formulations targeting specific pathogens.
- The methodology proposed by EUCAST for the determination of antimicrobial activity of antibiotics and other substances is limited when applied to NeoGiANT extracts and formulations. Therefore, the modification of these protocols should be considered in order to enable the proper evaluation of compounds with a more complex chemical nature

WP4 Assessment of the antimicrobial formulation for feeding at small scale

1. Scientific & technical achievements

■ What were the main scientific and technical achievements

Work package 4 successfully developed and validated functional feed and/or drinking water supplements for multiple species: fish, calves, piglets, and poultry. Safety, efficacy, and species-specific applicability were systematically evaluated, and scalable production methods were established.

1. Calves

Supplementation in feed with 1500 ppm polyphenols was safe and palatable, with no effect on growth performance.

Supplementation (1500 ppm polyphenols) has not shown to be protective against experimentally induced intestinal colonisation of *E. coli* O157 in calves.

2. Piglets (around weaning)

■ In vitro testing

The effects of polyphenol extracts on cell viability, permeability, and gene expression of genes of pro- and anti-inflammatory cytokines were assessed per solvent at specific concentrations.

■ Effects on growth performance

Supplementation in feed of 750 and 1500 ppm polyphenols (ACS) were safe for post-weaned piglets, reduced diarrhea, improved intestinal villi length, and may enhance nutrient absorption, weight gain and welfare.

■ Infection trial with *E. Coli* O147 and O149

A high-polyphenol diet (1500 ppm; ACS) improved immune response, reduced diarrhea and *E. coli* concentration, enhanced weight gain and feed efficiency, lowered pathological scores, and supported intestinal health in infected piglets (not sign.)

■ Trial with supplementation 14 days before and 14 days after weaning

Polyphenol supplementation was associated with lower leukocyte counts, reduced activities of

hepatic injury markers (ALT and AST), higher serum albumin concentrations, increased ALP activity, and a lower incidence of hepatic micro-vesicular steatosis despite higher circulating lipid concentrations after weaning. These changes suggest that polyphenols may support hepatic metabolic adaptation, protein utilisation, bone growth, and lipid handling during the suckling-to-weaning transition.

In addition, changes in mineral-related parameters, particularly iron and calcium, appeared transient and physiologically driven, reflecting altered mineral partitioning, erythropoietic adaptation, and skeletal growth during the suckling-to-weaning transition rather than overt mineral deficiency, although an inhibitory effect of polyphenols on iron absorption cannot be excluded. In parallel, adaptive alterations in jejunal morphology and gene expression indicate support of intestinal homeostasis, without adverse effects on growth performance or overall health.

3. Broilers

No clear (significant) positive effects on zootechnical performance were observed with feed supplementation (max. 1500 ppm polyphenols; ACS).

Drinking water supplementation produced better results, with varying (and often significant) effects depending on dosage (2 ppm and 10 ppm polyphenols; LLN) and broiler age (grower vs. finisher phase). Several pen trials were conducted, with treatment and control groups always housed within the same house. Low and high dosages were also alternated to allow replication of findings despite possible house effects. In addition, a large-scale field trial was carried out at house level. Supplementation of drinking water with the NeoGiANT extract showed promising potential to support broiler performance, even in flocks with already high baseline health and productivity. While effects varied depending on environmental and management conditions, the results highlight opportunities to optimize dosage and timing to

achieve consistent benefits. Significant increases in propionic, n-valeric, and isocaproic acids were observed in the crop of birds receiving 10 ppm polyphenols. These early increases in SCFA production may enhance beneficial microbiota activity, improve intestinal fermentation and antimicrobial effects, and ultimately support better nutrient absorption and productive performance. Furthermore, observed impacts on the microbiome and immune system suggest additional ways the extract could contribute to flock health, making it a promising strategy for future poultry production.

4. Aquaculture

Different concentrations and solvents (ELN, PLN, ACA at 45, 200 and 450 ppm polyphenols) were tested employing zebrafish (*Danio rerio*) as animal model. There were no evident differences between dietary treatments in all growth parameters and final survival for each trial separately. Moreover, the experimental diets did not modulate the oxidative stress (CAT, GR, LPO) response of the zebrafish. RNAseq revealed a total of 3100 differentially expressed genes (DEG) particularly related with the immune system and oxidative status/stress.

European seabass fed PLN450 had a higher growth performance (final body weight, weight gain, daily growth index, feed efficiency, and protein efficiency ratio) and hepatic antioxidant activity (SOD, catalase, GPX, GR) (not significant).

European seabass fed PLN450 and ACA450 diets managed to resist better to an infection with *Vibrio harveyi*, having higher survival rates (not significant).

Atlantic salmon fed ACA1500 showed significantly better gill health ($p=0.028$) and histology scores ($p=0.0421$) after infection with *Neoparamoeba perurans*.

Turbot fed PLN450 and ACA450 presented higher final body weight, weight gain, daily growth index, feed efficiency, and protein efficiency ratio, than the CTR diet (not significant). Diets supplemented with PLN900 and ACA900 showed the lowest final

body weight and weight gain. This may indicate that using concentrations between 450 and 900ppm of the NeoGiANT extracts may induce greater growth performance and feed utilisation efficiency in turbot.

Turbot fed diets PLN450 and ACA450 showed improved resistance to *Vibrio anguillarum* infection, with higher survival rates (66,7% and 53,3%, respectively) than the control (46,7%)(not significant).

For Rainbow trout, no differences were observed in intestinal histology, microbiota diversity, or blood biochemical parameters among dietary treatments. However, fish fed the ELN450 diet showed significantly higher catalase (CAT) activity compared to control diets (C1 and C2), while those fed ACA450 and PLN450 exhibited increased superoxide dismutase (SOD) activity relative to C1. Gene expression analysis revealed a significant upregulation of $il-1\beta$ in fish fed the ACA-supplemented diet (450 ppm) compared to CTR2 (final sampling) and ELN450 groups, whereas $il-6$ and $tnf-\alpha$ expression levels were not affected by dietary treatments. Regarding *Flavobacterium psychrophilum* prevalence and survival, fish fed the PLN extract showed the highest infection prevalence but also the highest survival rate among the tested extracts. Although no statistically significant differences were detected, these results suggest a potential protective effect of the PLN extract against the infection.

■ What were the main scientific and technical achievements

Technically, scalable and safe production processes for extracts and formulations were established for multiple animal species and application routes:

Diets for pigs and poultry were successfully prepared on a small scale using a solid aqueous extract (after acetone volatilization) (ACS) at various concentrations ranging from 200 to 1500 ppm of polyphenols. The results demonstrated that the target polyphenols present in the extracts were also detected in the diets

WP4 Assessment of the antimicrobial formulation for feeding at small scale

containing these extracts and that they remained stable during storage for at least one year.

Diets for 3 fish species (zebrafish, European seabass and trout) at small scale were successfully prepared using different extracts (PLN, ELN, ACA) at different concentrations (45, 200, 400, 900, 1500 ppm polyphenols). The extract was mixed with the other ingredients before pelleting. It has been demonstrated that target polyphenols (those present in the extracts) were also present in the diets containing the extracts.

The salmon feeds were produced with solid extract (ACS) via dry coating (powder mixing) and with liquid extract (ACA) via liquid spraying (surface coating).

For poultry, a liquid drinking water supplement was prepared from liquid extract (LLN) and optimized for use under conventional poultry farming conditions.

■ How were they addressed?

Scientific achievements were addressed through systematic screening of extracts, solvents, and concentrations, followed by formulation optimisation and efficacy testing. Safety, efficacy, and species-specific applicability were evaluated using biological performance indicators (including biochemical and haematological parameters and histological analyses), microbiome analysis, and immune-related parameters.

Technical challenges were addressed by developing and validating different extraction, formulation, drying and delivery methods tailored to feed and water applications, ensuring stability, homogeneity, and compatibility with existing feeding systems.

■ What key methodologies or technologies were developed?

- Selection and validation of safe, food-grade solvents with optimal extraction efficiency.
- Development of species- and application-specific formulations for in-feed and drinking water use.

- Scalable production methods suitable for industrial feed and water supplementation.
- Determination of safe and effective polyphenol concentrations for each species and application.
- Understanding how product formulation, dose, age, and environmental/management conditions affect health, performance, and immune/microbiome responses.
- Development of a standardized dissection and sampling protocol for the zebrafish (*Danio rerio*) experimental model, with special emphasis on samples intended for histopathological evaluation and molecular analyses (<https://doi.org/10.1089/zeb.2024.0184>).
- Development, validation, and standardization of analytical methods for the detection and quantification of target polyphenols and their metabolites in feed and in biological samples, including serum, crop content, and caecal content.

2. Key Environmental Impact

■ What are the most significant contributions of your WP to NEOGIANT?

WP4 generated robust, species-specific evidence on the safety, efficacy, and limitations of polyphenol-based feed and drinking water supplements in calves, pigs, poultry, and aquaculture species, while also contributing to the environmental sustainability objectives of NEOGIANT through data supporting improved animal resilience, and potential reductions in production-related environmental footprint. Key contributions include:

- Safety validation of polyphenol supplementation across calves, piglets, poultry, and fish at multiple inclusion levels and via different delivery routes.
- Demonstration of species- and route-dependent efficacy, showing that

- biological effects strongly depend on dosage, solvent, formulation, and application method (feed vs. drinking water).
- Proof of concept for health-promoting effects, particularly in piglets and aquaculture species, including reduced diarrhoea, improved intestinal morphology, enhanced immune response, antioxidant activity, and disease resistance.
- Identification of limitations, such as the lack of protective effects against *E. coli* O157 colonisation in calves and inconsistent zootechnical responses in broilers when supplemented via feed.
- Generation of comparative data across solvents and concentrations, supporting informed optimisation of formulations for different animal species and production systems.
- Improved animal health and resilience, which may decrease morbidity and mortality rates, leading to better feed efficiency and reduced resource wastage.
- Contribution to lower environmental footprint, as improved feed conversion efficiency and disease prevention can reduce overall feed input per unit of animal product, thereby lowering associated greenhouse gas emissions (including CO₂ equivalents) and nutrient excretion.
- Support for more sustainable production systems, by promoting natural, plant-based additives as alternatives to synthetic compounds, potentially reducing reliance on resource-intensive or environmentally burdensome inputs.

Overall, WP4 provided critical biological validation data linking formulation characteristics to animal health, performance, and resilience outcomes, thereby supporting downstream product development and application strategies within NEOGIANT, while also contributing to more sustainable livestock and aquaculture systems with the potential to reduce antimicrobial reliance

and lower the environmental footprint of animal production.

■ How can these results be used in future projects or policies?

The results of WP4 provide a strong foundation for both future research initiatives and policy-relevant developments:

- Guidance for targeted formulation development: The demonstrated species-, dosage-, and delivery-specific responses can inform future R&D projects focused on precision nutrition and tailored functional feed solutions.
- Support for alternative strategies to antimicrobial use: Positive effects on gut health, immune response, and disease resistance support the further development of polyphenols as non-antibiotic health-promoting feed additives.
- Input for regulatory and policy discussions: Safety data across species and production stages contribute to evidence-based assessment of plant-derived feed additives within EU feed and animal health policies.
- Design of future large-scale and long-term trials: Identified variability in efficacy highlights the need for refined dosing strategies, standardized trial designs, and longer-term field validation in future research projects.
- Contribution to sustainable livestock and aquaculture systems: Improved animal resilience, feed efficiency, and health resilience align with EU objectives on sustainability, reduced environmental impact, and animal welfare.

WP4 Assessment of the antimicrobial formulation for feeding at small scale

3. Challenges and Lessons Learned

■ Were there any unforeseen factors that impacted the research?

Several unforeseen factors influenced the progress and outcomes of the research. Initial trials did not immediately show clear or consistent effects, indicating that the originally selected dosages were not optimal. A formal dose-response study was not included in the initial experimental plan, which required iterative adjustment of both extract concentration and formulation during the project.

In addition, substantial optimisation of the extracts was necessary, including adaptation from feed to drinking water applications, as efficacy differed between delivery routes.

Another key challenge was that many trials were conducted in animals with very good baseline health status. Under such conditions, the effects of natural bioactive compounds are difficult to objectify, as measurable benefits often only become apparent under physiological or infectious stress.

A major limitation in the fish trials was the toxicity of the different extracts when administered by bath. These treatments proved highly harmful to all tested fish species, even at low extract concentrations in the water.

■ How did these challenges influence project outcomes?

These challenges led to a more stepwise and evidence-driven approach, requiring additional trials to optimise formulation, dosage, and application strategy before progressing to large-scale field studies. Rather than directly implementing large field trials, the project prioritised proof-of-efficacy under controlled conditions to avoid inefficient use of resources.

For example, in piglets, the strategy was adjusted from an initial large field trial in post-weaning diarrhoea (PWD) settings to a controlled challenge model, with supplementation starting before challenge (weaning). This approach

allowed clearer evaluation of biological effects and improved understanding of timing and conditions under which supplementation is most effective.

Overall, these lessons highlighted the importance of dose optimisation, appropriate challenge models, and phased validation when developing functional feed and water supplements based on natural products.

For the Rainbow trout preventive trials, bath administration of the extracts showed unexpectedly high toxicity in preliminary tests, indicating that this approach was unsafe for this species. Consequently, the planned experiment was cancelled and replaced with a redesigned study using Rainbow trout eggs experimentally infected with *Saprolegnia* spp. Additionally, a new zebrafish trial was planned using a solid formulation of the extract.

4. Opportunities / Suggestions

■ What potential opportunities emerged from the project that could be further explored?

- Polyphenol supplementation demonstrated species-, dose-, and route-specific effects, with clear benefits in piglets and aquaculture, and context-dependent (environmental and management factors) benefits in broilers.
- Evidence of improved growth performance, gut health, immune response, antioxidant activity, and disease resilience suggests potential for natural feed additives to enhance animal health and welfare.
- Lower extract concentrations sometimes outperformed higher doses in aquaculture, highlighting opportunities for cost-efficient, high-effect formulations.

Are there recommendations for future research, industry applications, or policy developments?

■ For research:

Conduct formal dose-response studies across species to define optimal concentrations and timing.

Use controlled challenge trials before large-scale field trials to ensure measurable effects.

Standardize methodologies for evaluating efficacy, bioavailability, and stability of natural additives across different production systems.

Explore mechanisms of action at molecular and physiological levels to better understand species-specific responses.

■ For industry:

Develop species-specific formulations, apply adaptive dosing relative to stress periods, and select appropriate delivery methods (feed vs. water).

Implement quality control systems to ensure consistency in active compound concentrations, particularly given natural variability in plant-based additives.

■ For policymakers:

Leverage safety and efficacy data to support regulatory assessment and promote sustainable livestock and aquaculture practices, including reduced antimicrobial use and improved animal welfare.

Develop clear, harmonized regulatory frameworks specifically addressing the use of natural additives in animal production.

Establish standards for quality, purity, labeling, and claims to prevent variability and misleading marketing.

Encourage evidence-based approval processes that balance innovation with consumer safety and environmental protection.

Support policies that incentivize alternatives to antimicrobials while ensuring that natural additives meet scientifically validated efficacy and safety criteria.

5. Please provide three short bullet-point highlights summarising the most relevant or impressive results achieved in your Work Package

- **Piglets:** High-polyphenol supplementation in-feed (1500 ppm) improved gut health, immune response, reduced diarrhoea, and supported weight gain and feed efficiency under challenge conditions.
- **Aquaculture:** Optimal polyphenol concentrations enhanced growth performance, antioxidant activity, and disease resilience in Seabass, Salmon, Rainbow trout and Turbot.
- **Broilers:** Drinking water supplementation showed context-dependent benefits, with supplemented groups achieving higher final body weights under specific climate, environmental, and management conditions. The effects depended on the dose and age of the broilers, highlighting dose- and age-specific efficacy.

WP5 Assessment of the antimicrobial formulation for animal diseases treatment products

1. Scientific & technical achievements

WP5 successfully evaluated the in vivo antimicrobial performance of extract-based optimized formulations for animal treatment application. The WP generated evidence across three animal infections case: mastitis in dairy cattle, skin infection in pigs, and infectious disease contexts in aquatic animals, by progressing from safety/ tolerance to in vivo efficacy assessment.

- **What were the main scientific and technical achievements?**
- **Formulation development and readiness for animal treatment**

Optimized formulations were prepared and assessed for suitability in animal treatment contexts, including topical, intramammary, and aquatic applications.

■ **Bovine mastitis model**

Extract-based intramammary injection formulations (ACA) were evaluated in an in vivo cattle mastitis model, assessing safety, animal tolerance, and antibacterial performance under controlled experimental conditions against the major pathogens that cause mastitis in cattle:

Streptococcus uberis and *Staphylococcus aureus*.

The challenge models showed a high and reproducible infection rate, confirming its suitability for assessing treatment effects. The in vivo intramammary administration of the grape-extract formulation in lactating dairy cows demonstrated acceptable safety but limited therapeutic efficacy in experimental mastitis models. In the *Strep. uberis* model, some trends were observed after repeated treatment (clinical evolution, milk quality, and bacterial counts), however these were not statistically significant.

While prior in vitro studies indicated a strong antimicrobial potential of the extracts, these effects were not translated into clear in vivo efficacy under the conditions tested.

■ **Porcine skin infection model**

The efficacy of selected bioadhesive formulations (ELN) was tested in an experimental porcine model of *Staphylococcus hyicus*, infected skin wounds, demonstrating feasibility of extract-based approaches for topical antimicrobial treatment. Across two independent trials, treatment with extract-based formulations resulted in lower bacterial counts compared to untreated controls and visible improvements in wound appearance, including reduced scab formation. Higher concentrations of polyphenols content formulations showed stronger effects, supporting dose-dependency in topical applications. Importantly, the model allowed effective down-selection of formulation prototypes and demonstrated that extract-based approaches can exert antibacterial effects in a clinically relevant porcine skin infection context.

No major safety concerns were observed, confirming feasibility for local treatment strategies

■ **Aquatic animal disease models**

Initial prototypes were assessed in in vivo aquatic models, progressing from zebrafish screening to relevant aquaculture species, to evaluate antibacterial performance, safety and tolerance, and practical applicability.

WP5 implemented a stepwise aquatic evaluation strategy, starting with zebrafish pre-screening to assess tolerance and guide formulation selection, followed by translation attempts to commercially relevant species. While zebrafish screening supported initial progression, subsequent rainbow trout and North Atlantic salmon bath exposure revealed marked toxicity even at low concentrations, leading to early ethical termination of the bath treatments.

This negative outcome represents a major technical achievement of WP5 in aquatic infections models, as it clearly demonstrated that bath treatment of extract-based formulations is not a viable route under practical aquaculture conditions.

A redesigned feeding-based in vivo assessment in trout showed no statistically significant improvements in growth performance or survival, although survival was numerically lower in control groups than in extract-fed fish. In contrast, feeding-based supplementation in Atlantic salmon resulted in a significant improvement in gill health and histopathology scores following infectious challenge, demonstrating that oral delivery of extract-based formulations can provide measurable health benefits in specific aquatic species and disease contexts.

■ **How were they addressed?**

All achievements were obtained using a controlled and ethically sound in vivo approach, with assessment steps in which safety and tolerability were evaluated prior to efficacy testing, and predefined stop/continue criteria were applied when well-being or feasibility limits were exceeded. WP5 deliberately combined positive signals with negative outcomes, ensuring that the results could be used for informed decision-making rather than overly optimistic interpretations.

■ **What key methodologies or technologies were developed?**

WP5 delivered validated in vivo infection models for cattle, pigs, and aquatic species, alongside practical decision frameworks integrating safety, efficacy, and feasibility. These methodologies provide practical tools for future projects aiming to develop non-antibiotic veterinary treatment products, while minimising animal use and development risk.

2. Key Environmental Impact

■ **What are the most significant contributions of your WP to NEOGIANT?**

Work Package 5 contributed to NEOGIANT's objectives by generating in vivo evidence for extract-based antimicrobial alternatives intended to reduce the use of conventional antimicrobials drugs in animal health. By evaluating these approaches in clinically relevant animal disease

models, WP5 clarified where such alternatives can realistically contribute to disease management and where they are not environmentally or practically viable. A key contribution of WP5 is the early identification of unsuitable delivery routes, particularly in aquaculture, where bath exposure was shown to pose tolerability and welfare risks.

Overall, WP5 contributed validated in vivo knowledge on extract-based antimicrobial approaches derived from sustainably sourced plant by-products, supporting the development of environmentally responsible veterinary treatment strategies. By identifying application scenarios with controlled exposure and clear biological benefit, WP5 helped align NeoGIANT outcomes with goals of reduced antimicrobial use, improved animal welfare, and lower environmental risk, thereby strengthening the project's contribution to sustainable animal production systems.

■ **How can these results be used in future projects or policies?**

WP5 results can guide future projects toward species- and application-specific development of novel non-antibiotic alternatives in animal health solutions, with a focus on delivery routes that improve treatment feasibility while ensuring animal safety and welfare. The validated in vivo models and stepwise evaluation approach can be reused to prioritise effective strategies early and reduce unnecessary testing and supporting the 3Rs principles.

From a policy perspective, WP5 supports evidence-based antimicrobial reduction strategies by showing that natural extract-based alternatives require targeted use and rigorous in vivo validation to deliver animal welfare benefits. The results contribute to EU objectives on sustainable livestock and aquaculture production, antimicrobial stewardship, and One Health.

WP5 Assessment of the antimicrobial formulation for animal diseases treatment products

3. Challenges and Lessons Learned

■ Were there any unforeseen factors that impacted the research?

Several unexpected factors influenced the progress and outcomes of WP5. A key challenge was the variability between animal species and treatment routes, particularly regarding safety and animal tolerance to extract-based formulations. Notably, toxicity observed during bath treatment in aquatic animals and inflammatory responses following intramammary application in dairy cattle highlighted the importance of delivery context. These issues required early termination, redesign, and optimisation of some in vivo studies, including grape-extract formulations, to ensure animal welfare and the feasibility of treatment approaches.

Additional challenges arose from the complexity of in vivo disease models, namely in mastitis infections models, where local inflammation, baseline animal health, and practical treatment constraints influenced outcomes. This highlighted the need for adaptive study designs and cautious interpretation across species and application routes.

■ How did these challenges influence project outcomes?

These challenges led to a more stepwise and adaptive evaluation strategy, including study redesign and formulation optimisation when safety or feasibility issues arose. WP5 therefore prioritised controlled decision-making and animal risk reduction over broad efficacy claims, strengthening the robustness and relevance of the outcomes.

Refinements of the formulations, administration strategies, and evaluation protocols across the different in vivo disease models enabled more reliable assessment of efficacy and safety without compromising overall objectives. The resulting additional experiments and methodological improvements strengthened disease models, clinical scoring, and microbiological endpoints, highlighting the importance of dose and

formulation optimisation, model-specific validation, and phased development for non-antibiotic animal treatment products based on natural compounds.

4. Opportunities / Suggestions

■ What potential opportunities emerged from the project that could be further explored?

The WP5 highlighted model- and disease-specific responses to natural extract-based treatments, supporting the opportunity to tailor non-antibiotic therapies to specific species, indications, and routes of administration. The establishment of robust in vivo models for mastitis, porcine skin infection, and aquatic species provides a platform for further optimisation and comparative testing of natural treatment strategies.

Observed dose- and application-dependent effects indicate opportunities to develop cost-efficient, targeted formulations that balance efficacy, safety, and practical field use while supporting reduced antibiotic reliance.

■ Are there recommendations for future research, industry applications, or policy developments?

Future research should focus on species- and disease-specific optimisation of extract-based treatments, including dose-response evaluation and refinement of feasible delivery routes, with controlled in vivo challenge models prioritised before large-scale field trials.

For industry, WP5 supports the development of targeted, application-specific non-antibiotic treatments, with early integration of safety, animal welfare, and practical feasibility into product design.

From a policy perspective, the results reinforce evidence-based antimicrobial reduction strategies, highlighting the importance of rigorous in vivo validation and realistic implementation pathways when promoting natural extract-based alternatives in sustainable animal health frameworks.

5. Please provide three short bullet-point highlights summarising the most relevant or impressive results achieved in your Work Package

- Cattle (mastitis): Established and applied a robust in vivo mastitis challenge model, demonstrating feasibility of extract-based intramammary treatment approaches while identifying formulation- and route-dependent tolerance limitations critical for further development.
- Pigs (skin infection): Demonstrated a strong antibacterial activity of

- extract-based formulations in a controlled porcine *Staphylococcus hyicus* skin infection model, supporting the potential of targeted, topical non-antibiotic treatments.
- Aquatic animals: Provided decisive evidence on species- and route-specific feasibility, ruling out bath treatments due to safety constraints while identifying oral delivery as a viable approach with improved gill health outcomes in Atlantic salmon.



WP6 Assessment of the antimicrobial formulation for sperm preservation

1. Scientific & technical achievements

■ What were the main scientific and technical achievements?

WP6 focused on testing antimicrobial formulations for sperm preservation. WP partners tested sperm extender formulations provided by WP2, delivering usable concentration ranges for the natural extracts across the four species groups: fish, poultry, pig, and cattle.

After obtaining these ranges, the WP partners refined specific extract concentrations and their effects. This study included testing extracts from the improved methodology developed in WP2, including aqueous and solid extracts.

The WP also provided estimates of the antimicrobial effects of the natural extracts in the different formulations in the absence of antibiotics. Additionally, WP6 conducted basic research on sperm analysis and on the effects of polyphenols on spermatozoa, not only in complex polyphenol mixtures but also individually and at different concentrations.

Therefore, WP6 delivered sperm extender prototypes at small scale, enabling practical testing and commercial validation in WP8.

Additionally, the WP delivered improved methodology for sperm assessment and information about sperm microbiology.

■ How were they addressed?

For the first part, spermatozoa from each species group were tested with a wide range of extract concentrations, seeking to detect toxic effects deriving from the extracts' composition or the organic solvents (expected at high concentrations). The compatibility of the sperm-extender-extracts compositions was also defined at this point, complementing WP2 findings on the formulation stability.

Once the WP defined species-specific sperm-safe concentration ranges, the partners tested the suitability of the selected formulations for refrigerated and cryopreserved sperm storage. This step included sperm quality and

microbiological analyses at different time points (hours or days for refrigerated storage and post-thawing status for cryopreserved storage). Additional tests were developed for refining the knowledge acquired on the extracts' effects, especially dose-response matrices for the individual polyphenols. In the pig species, these assays were complemented with infection challenge tests (spiking semen samples with bacteria of interest).

■ What key methodologies or technologies were developed?

- In vitro sperm toxicity assays to determine safe concentration ranges of natural extracts.
- Sperm quality analysis, both cellular and molecular, to determine the effects of the formulations on sperm physiology and metabolism.
- Enhanced microbiological analyses for semen samples in the four species groups.
- Microbiological infection tests in semen extenders containing natural extracts.
- Functional evaluation of antimicrobial activity within formulated semen extender doses.

2. Key Environmental Impact

■ What are the most significant contributions of your WP to NEOGIANT?

This WP was critical in enabling NeoGiANT to impact animal reproduction by testing the efficiency of the sperm extender formulations and assessing their effects on sperm quality and microbiology. WP6 contributed critical scientific evidence on the limitations and advantages of replacing antibiotics with natural extracts in semen extenders.

Considering NeoGiANT's structure and viability, WP6 bridged the knowledge gained in WP2 and WP3 regarding formulation development and antimicrobial analyses with that in WP8 regarding the practical and commercial testing of the new sperm extenders. WP6 used WP2 and WP3

deliverables on animal reproduction, delivering sperm extender prototypes to WP8 to continue this line of work and select those appropriate for commercial validation.

Overall, WP6 contributes validated formulations containing natural extracts sustainably obtained from plant refuse, which may be considered for applied or commercial use after testing the spermatozoa's fertility in the compositions. This way, it supports the development of novel antimicrobial products in animal production based on natural extracts derived from agrifood byproducts as raw materials

■ How can these results be used in future projects or policies?

They support evidence-based decision-making in developing sustainable alternatives to antibiotics and can inform future policies by highlighting the need for rigorous toxicity and efficacy assessments before implementation.

WP6 sets an example for future projects seeking to replace antibiotics with natural extracts in animal reproduction. Whereas WP6 has focused on the sperm cell, studies on other aspects of animal reproduction or on assisted reproductive techniques (e.g., oocyte or embryo preservation, or in vitro embryo production) could benefit from the knowledge gained when applying natural extracts with similar objectives.

Considering the general trend towards reduced antibiotic use in animal reproduction, and even a ban, at least in some applications, WP6 results could guide the formulation and implementation of new rules. These results could also inform new guidelines on animal reproduction and the drafting of future research programs aimed at replacing antibiotic use in this area.

3. Challenges and Lessons Learned

■ Were there any unforeseen factors that impacted the research?

WP6 partners found a high sensitivity of spermatozoa to some of the extracts' vehicles

and to some of the extracts' compositions. Although a relatively high sensitivity of spermatozoa to chemicals (in this case, organic solvents and polyphenols) was anticipated, it was higher than expected, and we were forced to redesign the work plan, especially for these species. It implied extending WP6's duration and delaying the deliverables for some months.

■ How did these challenges influence project outcomes?

Although this factor impacted the research by requiring additional tests and experiments, the WP6 work plan could be successfully modified, delivering the required deliverables on time and without significantly affecting WP8's performance (which partly depended on WP6). In fact, the modifications resulted in additional outputs from the design and execution of new experiments (e.g., dose-effect studies on individual polyphenols and bacterial spiking trials).

This challenge motivated us to adjust the concentrations of the extracts in the sperm extenders. However, this high sensitivity of spermatozoa from very diverse species to natural extracts is, in itself, a worthy result with scientific and practical value for subsequent projects. Moreover, the additional research increased the number of formulations tested and the information on sperm physiology and semen microbiology obtained in WP6.

4. Opportunities / Suggestions

■ What potential opportunities emerged from the project that could be further explored?

WP6 found a differential effect of different polyphenols, at least in the pig and cattle species. Therefore, natural extracts could be classified according to their suitability as supplements for sperm extenders, enabling the identification of manufacturing processes more suited to producing sperm-friendly extracts that could result in enhanced formulations, while ensuring sustainable extract production.

WP6 Assessment of the antimicrobial formulation for sperm preservation

1. Scientific & technical achievements

■ What were the main scientific and technical achievements?

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After obtaining these ranges, the WP partners refined specific extract concentrations and their effects. This study included testing extracts from the improved methodology developed in WP2, including aqueous and solid extracts.

The WP also provided estimates of the antimicrobial effects of the natural extracts in the different formulations in the absence of antibiotics. Additionally, WP6 conducted basic research on sperm analysis and on the effects of polyphenols on spermatozoa, not only in complex

polyphenol mixtures but also individually and at different concentrations.

Therefore, WP6 delivered sperm extender prototypes at small scale, enabling practical testing and commercial validation in WP8.

Additionally, the WP delivered improved methodology for sperm assessment and information about sperm microbiology.

■ How were they addressed?

For the first part, spermatozoa from each species group were tested with a wide range of extract concentrations, seeking to detect toxic effects deriving from the extracts' composition or the organic solvents (expected at high concentrations). The compatibility of the sperm-extender-extracts compositions was also defined at this point, complementing WP2 findings on the formulation stability.

Once the WP defined species-specific

WP7 Effects on the gastrointestinal tract and the immune system

Scientific & technical achievements

■ What were the main scientific and technical achievements

In vivo trials performed in pigs, poultry, calves, and fish demonstrated that dietary supplementation with grape polyphenolic extracts did not induce major alterations in intestinal microbiota composition or overall microbial diversity, and thus the tested extracts have not compromised gut microbial homeostasis under the experimental conditions. The relative abundance of dominant bacterial taxa, including relevant enteric pathogens (*Salmonella*, *Escherichia-Shigella*), mainly remained stable across species in livestock. Notably, a reduction in some potentially pathogenic bacterial groups (e.g. *Pseudomonas*, *Helicobacteraceae*) was observed in certain trials. In vivo fish trials were conducted in European seabass, zebrafish and trout, and grape polyphenolic extracts have not induced major alterations in intestinal microbiota composition, in overall microbial diversity or in the relative abundances of the tested primary fish pathogens (such as *Flavobacterium*, *Vibrio* and *Aeromonas*). In a dose finding study in European seabass grape extracts significantly reduced the relative abundance of the opportunistic pathogen *Stenotrophomonas*. Furthermore, a database was established to demonstrate the effect of different plant extract on the intestinal microbiota. Plant extracts possess a wide range of chemical compounds (e.g. polyphenols), and their effects on microbial populations can differ considerably depending on the plant source, extraction technique, dosage, and animal species. Studies on the gut microbiota should therefore be conducted whenever an additive or substance intended for animal feed may potentially exert a notable antimicrobial effect at its target inclusion level. Based on the obtained findings, a database was created that summarizes the effects of different grape extracts, and the data can be publicly and freely accessed by stakeholders.

Comprehensive immunological assessments covered cellular, humoral, and functional immune parameters, as well as antioxidant status. Results

consistently demonstrated measurable, species-specific immunomodulatory effects. In poultry, supplementation induced dose-dependent modulation of immune cell subpopulations and cytokine profiles, leading to enhanced innate immune activity while preserving immune balance. In cattle, high-dose polyphenol supplementation resulted in reduced circulating pro-inflammatory cytokine levels, indicating anti-inflammatory potential. In fish, polyphenol-rich diets modulated the expression of immune- and antioxidant-related genes, supporting immune homeostasis and improved stress resilience. In pigs, grape polyphenol supplementation selectively influenced lymphocyte subpopulations during experimental *Escherichia coli* infection, suggesting enhancement of cell-mediated immunity, while no adverse effects on vaccine-induced immune responses were observed. Overall, the project results demonstrate that grape polyphenols represent a sustainable, natural dietary strategy with the potential to support gut health and immune resilience in livestock and aquaculture systems, aligning with EU objectives on reduced antimicrobial use.

■ How were they addressed?

To analyze the gut bacterial composition, NeoGiant utilized both amplicon metagenomic sequencing and culture-based techniques.

An additional main objective was to characterise immune-physiological responses induced by dietary polyphenol supplementation, with a focus on disease resistance, antioxidant capacity, and modulation of inflammatory processes.

■ What key methodologies or technologies were developed?

For the metagenomic approach, gDNA was isolated from animal fecal or intestinal samples and sequenced using the Illumina MiSeq platform targeting the 16S rDNA V3-V4 region. These sequences were processed through the NCBI Nephel QIIME2 pipeline and compared against the SILVA reference database to identify bacterial taxa. Statistical significance for any observed

shifts in microbial abundance was determined by IBM SPSS Statistics software using the Kruskal-Wallis test with Bonferroni adjustments. The reproducibility of the amplicon metagenomic analyses were confirmed by use of Kraken software. In parallel, culture-based analyses measured the colony-forming units (CFU/g) of both total aerobic and anaerobic bacteria, as well as specific pathogens like *Salmonella* and *E. coli*. This involved serial dilutions of homogenized samples and the use of spot-plating techniques on various selective bacteriological agar media. While metagenomic data provides a broad overview of the community, the culture-based results offer specific counts for viable bacteria.

Depending on the target animal species, the effects of polyphenols on the immune system were evaluated using different methodological approaches, with a predominant focus on ELISA assays (interleukins, TNF), gene expression analyses (mucins, heat shock proteins, TNF, protective enzymes), and flow cytometry analysis.

Key Environmental Impact

■ What are the most significant contributions of your WP to NEOGIANT?

Maintaining a balanced gut microbiota is essential for optimal digestion, immune function, and productivity in food-producing animals. Due to the chemical diversity of plant extracts and their species- and dose-dependent effects, it is critical to verify that their application does not induce dysbiosis or negatively affect beneficial microbial populations. At the same time, this multi-component nature represents a potential advantage, as the development of antimicrobial resistance is generally less likely or more difficult to occur compared to single-compound agents such as conventional antibiotics.

The gut microbiome and the immunological status of the host are highly interconnected, and their combined assessment represents a sensitive and reliable indicator of animal health, resilience, and environmental impact.

Consequently, integrated gut microbiota and immunological studies were conducted to assess whether NeoGiant grape polyphenolic extracts exhibit antimicrobial activity at the intended supplementation levels, while preserving microbial balance and immune homeostasis.

How can these results be used in future projects or policies?

In the NeoGiant project an in vivo inhibitory effect of white grape marc extracts on the relative abundances of various gut bacterial taxa that are prone to develop multidrug-resistance could be observed in certain trials, such as for the genera *Acinetobacter* and *Pseudomonas*. In a dose finding study in broilers with supplementation via drinking water the relative abundances of the genera *Acinetobacter* and *Pseudomonas* decreased significantly ($p < 0.05$ and $p < 0.01$, respectively) in the T4 grape extract-treated group (10 ppm total polyphenols in the drinking water) compared to the control group (0 ppm total polyphenols in their drinking water). *Acinetobacter* and *Pseudomonas* are two genera that are listed with critical and high priority, respectively, on the 2024 WHO list of bacteria for which new antibiotics are urgently needed (WHO, 2024), and thus could be targets of further projects with the aim to develop novel combinatory treatments against these pathogens.

The main objective of WP7 was to monitor changes in the gut microbiome and immunological status in response to supplementation with polyphenol-rich grape extracts. The results clearly demonstrated the strong interconnection and functional interdependence between these two systems, indicating that they should be jointly applied and evaluated in future research and development activities pursuing similar objectives. Both the gut microbiome and immunological parameters proved to be sufficiently sensitive indicators of health and resilience-related changes.

Collectively, the results support the use of grape polyphenols as sustainable, natural immunomodulators with the potential to improve

WP7 Effects on the gastrointestinal tract and the immune system

immune health and resilience in livestock and aquaculture systems, while contributing to antimicrobial resistance mitigation strategies and informing future research and policy development. Our results from the drinking water trials highlighted that a given polyphenol formulation can exert different effects depending on the age of the animals, thereby providing a broader range of potential applications in future use scenarios.

Challenges and Lessons Learned

■ Were there any unforeseen factors that impacted the research?

No such significant factors were observed. The sensitivity of the samples during transport was anticipated; however, for immunological measurements, the use of flow cytometry clearly required very short transport times. Special attention was given to this aspect, and whenever this could not be resolved otherwise, alternative methods were applied to compensate. As a result, this did not affect the overall success of the project.

■ How did these challenges influence project outcomes?

Not applicable

Opportunities / Suggestions

■ What potential opportunities emerged from the project that could be further explored? Further projects with the aim to develop against MDR pathogens novel combinatory treatments that include a white grape marc extract.

Are there recommendations for future research, industry applications, or policy developments? Future research is recommended to desing and test in vitro and in vivo novel combinatory

antimicrobial treatments that include a white grape marc extract. Related industry applications, or policy developments would need further studies based on the results already obtained by NeoGiant.

Please provide three short bullet-point highlights summarising the most relevant or impressive results achieved in your Work Package

- i. Dose-dependent promotion of beneficial gut bacteria: High-dose grape polyphenol supplementation (1500 ppm) in feed during a pre-weaning supplementation triall of pigs significantly increased Lactobacillus and Lactobacillaceae ($p < 0.05$), with a positive correlation to total polyphenol intake ($R = 0.447$, $p = 0.025$).
- ii. Targeted reduction of potentially pathogenic taxa: In broilers, 10 ppm grape polyphenols in drinking water significantly reduced Acinetobacter ($p < 0.05$) and Pseudomonas ($p < 0.01$), while ETEC-challenged pigs showed inhibition of Campylobacter/Campylobacteraceae at 1500 ppm total polyphenols.
- iii. Safe, sustainable immunomodulation without resistance: Grape polyphenol supplementation induced favourable microbiota and immune trends without disrupting overall diversity, and no tolerance or resistance developed in E. coli or Staphylococcus aureus in vitro against white grape extracts.



WP8 Ecodesign and pre-commercial validation at pre-industrial scale

1. Scientific & technical achievements

The project achieved significant scientific and technical progress across the development, validation and industrialization of NeoGiANT-based products, covering process design, sustainability optimization and multiple end-use applications in animal nutrition, aquaculture, animal reproduction and health.

A key technical achievement was the development of a digital twin of the up-scaled production process for NeoGiANT extracts, implemented using simulation software. This digital model generated technically feasible and industrially realistic process data, overcoming the limitations of laboratory-scale experimentation. The digital twin enabled the systematic optimization of the production process through the application of genetic and heuristic algorithms, simultaneously considering technical, environmental, economic and social performance. This approach demonstrated the feasibility of combining digital twins and multi-criteria optimization as a decision-support tool in early industrial scale-up phases, where empirical data are scarce, and contributed to bridging process engineering with sustainability assessment in early-stage of product development.

In the field of animal nutrition, NeoGiANT ingredients were successfully incorporated into feed formulations for different animal species. These formulations were scientifically validated to meet nutritional requirements and to comply with all relevant legal and safety regulations. Feed recipes were designed according to species-specific nutritional needs and tested for scalability, stability and safety under realistic storage conditions. Periodic sampling and analysis confirmed that the feeds maintained their nutritional quality and safety over time. All analyses were performed using official European reference methods, ensuring robustness, reproducibility and regulatory compliance.

For aquaculture applications, a pre-commercial NeoGiANT product based on grape marc extract was validated as a functional ingredient in fish

diets. Feeding trials with both marine and freshwater species (e.g. gilthead seabream, trout and amberjack) demonstrated that dietary fortification with this product reversed the negative effects associated with high inclusion levels of terrestrial plant proteins in aquafeeds. In addition, the functional ingredient positively modulated antioxidant defenses and the status of the intestinal mucosa. These results confirmed its functional role and applicability in aquaculture nutrition.

From an animal health perspective, NeoGiANT-derived products demonstrated antimicrobial activity in vitro, with ongoing analysis of in vivo performance. In parallel, the manufacturing process of the antimicrobial prototype was successfully industrialized, confirming its technical feasibility, consistency and scalability using current industrial production technologies.

In the area of animal reproduction, innovative semen extender formulations incorporating natural extracts as alternatives to conventional antibiotics were developed and validated. These formulations represented a novel solution for reducing antibiotic use in reproductive practices. Their performance was evaluated through a combination of laboratory experiments, in vitro assays and field fertility trials, demonstrating their practical and commercial potential. While no entirely new reproductive methodologies were created, existing artificial insemination and in vitro fertilization techniques were adapted and optimized to enable the evaluation of the newly formulated antibiotic-free semen extenders under both laboratory and field conditions. This optimization was essential to ensure reliable assessment of fertility performance while maintaining consistency with standard reproductive practices.

2. Key Impact

A major impact was achieved through the integration of eco-design and Life Cycle Sustainability Assessment (LCSA) at early, pre-commercial stages of product and process

development. This approach reduces the time and costs associated with scaling up laboratory prototypes to industrial production by anticipating technical, environmental, economic and social constraints before physical implementation. The sustainability assessments provided the first quantitative evidence of the potential sustainability advantages of NeoGiANT products compared to conventional alternatives, while also identifying key sustainability hotspots along the life cycle where further improvements can be targeted.

Beyond the project itself, the sustainability outcomes generated can contribute to future research and policy discussions by informing the definition of sustainability performance benchmarks or thresholds for novel bio-based products. They can also support the design of incentive schemes aimed at promoting more sustainable production pathways. In addition, the eco-design and LCSA framework applied is transferable to other research and innovation initiatives, particularly those addressing early-stage technologies where sustainability considerations are often not yet systematically integrated.

In the area of animal nutrition, the project demonstrated the capability to produce high-quality animal feed formulations that closely reflect what is used in real farming conditions. The validated feed mixtures confirmed that NeoGiANT ingredients can be reliably incorporated into practical, safe and nutritionally adequate feeds. This represents a concrete contribution to the project's overall objectives, demonstrating not only technical feasibility but also real-world applicability for farmers and feed producers. The methods used to design, test and assess these feeds are directly applicable to future projects and can be adopted in everyday practice to improve feed quality control, ingredient selection and safety management.

For aquaculture, the validation of a functional ingredient derived from grape marc extract represents a relevant impact in the context of sustainable animal production. The results

support the use of phytobiotics derived from plant by-products as functional alternatives to conventional compounds in aquafeeds. This opens opportunities for future research extending the evaluation to additional aquaculture species, such as crustaceans, and supports policy initiatives promoting circular economy approaches in aquaculture and animal production systems.

In the field of animal health, the generation of proof-of-concept results demonstrating antimicrobial activity provides a solid basis for continued field trials in target species. If confirmed through final laboratory and field data, these results pave the way for the development of effective alternatives to antibiotics, such as treatments for bovine mastitis, contributing to antimicrobial resistance mitigation strategies and more sustainable veterinary practices.

Finally, the project delivered critical field-scale validation for NeoGiANT-based reproductive products, providing the real-world fertility data required to assess their feasibility for early adoption. This validation was essential to determine whether product development could realistically progress beyond the experimental phase. By confirming or rejecting previous laboratory findings under operational conditions, the project ensured that only solutions with demonstrated practical applicability move forward, strengthening the robustness and credibility of the overall innovation pathway.

3. Challenges and Lessons Learned

In the sustainability analysis, one of the main challenges was the limited availability of reliable sustainability data for conventional products with functionalities comparable to those developed within the project. Environmental, economic and social data for benchmark products were often unavailable, fragmented or insufficiently transparent. This resulted in incomplete life cycle inventories for conventional alternatives, which reduced the robustness of comparative sustainability assessments. As a consequence, benchmarking exercises were able to provide indicative but not fully conclusive evidence of the

WP8 Ecodesign and pre-commercial validation at pre-industrial scale

sustainability improvements achieved. This highlights a broader structural limitation in sustainability assessment and underlines the need for improved data transparency and harmonized reporting for conventional products.

In the case of functional ingredients for aquafeeds, a key challenge related to the standardization of bioactive compounds in plant-derived extracts, particularly total polyphenols and specific marker compounds. While chemical standardization of the extracts could be achieved, additional unforeseen factors were identified that may influence biological effectiveness. Raw material variability and bioavailability issues emerged as critical aspects, as equivalent polyphenol content does not necessarily translate into equivalent biological responses. Differences in feed matrix composition, digestive enzymes and gut microbiota across fish species can significantly affect absorption and metabolism. This experience highlighted the importance of complementing chemical characterization with biological validation when developing functional ingredients for animal nutrition.

For reproductive applications, an important challenge was the high sensitivity of spermatozoa to the natural extracts used as alternatives to conventional antibiotics. This sensitivity required adjustments to experimental design and timing, including additional optimization steps to ensure safety and functionality. While some activities had to be rescheduled, these adaptations did not compromise the overall objectives.

On the contrary, they reinforced the importance of species-specific evaluation and careful dose optimization when introducing bioactive compounds into sensitive biological systems. For some species, data remain incomplete or inconclusive, indicating the need for further targeted research before drawing definitive conclusions.

4. Opportunities / Suggestions

One opportunity in the Life Cycle Sustainability field is the advancement towards more openly accessible and harmonized datasets on the environmental and social performance of products available on the market. Improved data availability would significantly strengthen sustainability benchmarking and decision-making for emerging bio-based solutions. This could be supported through the development of open data spaces, potentially based on anonymized product data to protect commercial confidentiality, and through stronger collaboration between industry, research institutions and public bodies. To ensure comparability and robustness, such datasets should be generated using harmonized and standardized assessment methodologies. For environmental performance, this includes the application of EU-endorsed frameworks such as the Product Environmental Footprint (PEF) and product-category-specific rules. Similar efforts are needed for the social dimension, including clearer methodological guidance, data structures and reporting practices.

In the area of animal feed production, the project highlighted opportunities to further optimize feed formulation and handling practices. Differences in particle size among ingredients can lead to heterogeneity and segregation during storage, potentially affecting nutrient distribution. Future work could focus on improving formulation design, processing techniques or ingredient pre-treatment to enhance homogeneity, as well as on the development of standardized sampling and homogenization protocols to ensure reliable and reproducible analytical results under both experimental and commercial conditions.

For aquaculture, the validation of a functional ingredient based on grape marc extracts opens multiple avenues for further exploration. Future research could extend the evaluation of these functional ingredients and associated bioactives to additional aquaculture species or groups, such as crustaceans, and to different developmental and rearing stages, including broodstock management, larval rearing, weaning, grow-out

sustainability improvements achieved. This highlights a broader structural limitation in sustainability assessment and underlines the need for improved data transparency and harmonized reporting for conventional products.

In the case of functional ingredients for aquafeeds, a key challenge related to the standardization of bioactive compounds in plant-derived extracts, particularly total polyphenols and specific marker compounds. While chemical standardization of the extracts could be achieved, additional unforeseen factors were identified that may influence biological effectiveness. Raw material variability and bioavailability issues emerged as critical aspects, as equivalent polyphenol content does not necessarily translate into equivalent biological responses. Differences in feed matrix composition, digestive enzymes and gut microbiota across fish species can significantly affect absorption and metabolism. This experience highlighted the importance of complementing chemical characterization with biological validation when developing functional ingredients for animal nutrition.

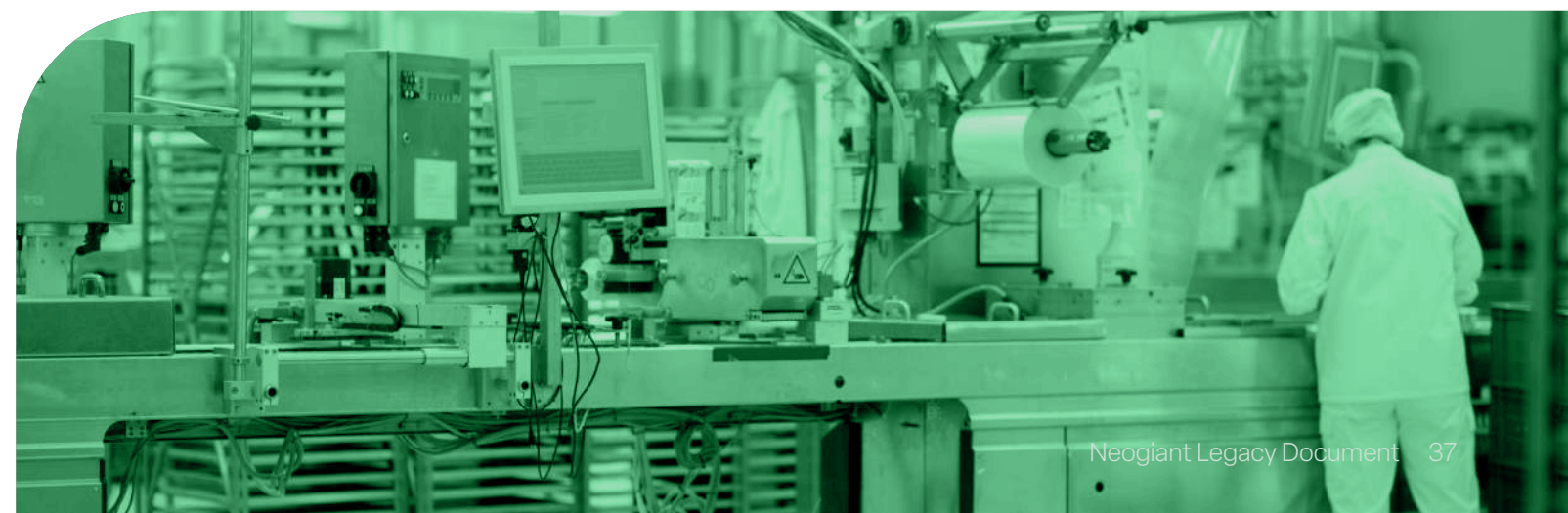
For reproductive applications, an important challenge was the high sensitivity of spermatozoa

to the natural extracts used as alternatives to conventional antibiotics. This sensitivity required adjustments to experimental design and timing, including additional optimization steps to ensure safety and functionality. While some activities had to be rescheduled, these adaptations did not compromise the overall objectives.

On the contrary, they reinforced the importance of species-specific evaluation and careful dose optimization when introducing bioactive compounds into sensitive biological systems. For some species, data remain incomplete or inconclusive, indicating the need for further targeted research before drawing definitive conclusions.

4. Opportunities / Suggestions

One opportunity in the Life Cycle Sustainability field is the advancement towards more openly accessible and harmonized datasets on the environmental and social performance of products available on the market. Improved data availability would significantly strengthen sustainability benchmarking and decision-making for emerging bio-based solutions. This could be





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