Recent Advances in Pharmaceutical Sciences III

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Preface

Despite the more than apparent pivotal role of scientific research as a key factor for boosting economic growth, in many countries R&D&i&t budgets are worryingly decreasing as a result of sustained economic recession. Notwithstanding several years of austerity measures, especially in southern European countries, the quality of the research carried out at the University of Barcelona is still keeping high standards, it being among the world top 100 universities in 14 out of the 30 subjects considered in the QS World University Ranking 2013, with a very meritorious 40th position in the particular subject of Pharmacy & Pharmacology.

This E-book compiles contributions in this field by internationally recognized research groups of the Faculty of Pharmacy of the University of Barcelona. The E-book consists of 12 chapters, including contributions in disciplines such as organic chemistry, pharmacognosy, food science, physical chemistry, biochemistry and molecular biology, plant physiology, botany, toxicology, physiology, health and environmental management, microbiology, and parasitology. Chapter 1 reports on the application of supramolecular chemistry for the preparation of different sorts of nanomaterials, namely gold nanoparticles and self-assembled monolayers, as promising tools in the clinical setting (Nanomedicine), emphasizing the paramount importance of the accurate design and selection of the components of the systems and the control of the self-assembly process as the determinants of the biofunctionality of these nanomaterials. Chapter 2 describes endeavors devoted to the discovery of novel antifungal agents belonging to a variety of phytochemical groups such as sesquiterpenes, triterpenes, flavonoids, lignans, fatty acids, and essential oils, which are based on an initial screening of ethnopharmacologically selected plants followed by bio-guided isolation of active constituents, with the hope that these compounds can overcome current therapeutic limitations due to the emergence of resistant fungal strains. Chapter 3 revises a multidisciplinary approach used to analyze the complex aroma of *cava*, the Spanish sparkling wine, which involves chemical, sensory, and olfactometric studies. In Chapter 4, the design of synthetic analogs of the membrane-active antibacterial peptide polymyxin B is presented, along with the development of artificial models of the outer layers of the target membranes in Gram-negative bacteria, namely monolayers, Langmuir-Blodgett films, and unilamelar vesicles, and a battery of biophysical methods to shed light on the structural determinants of their membrane action. Chapter 5 highlights the relevance of the modulation of the enzyme carnitine palmitoyltransferase 1 (CPT1), responsible for the catalysis of the rate-limiting step in mitochondrial fatty acid oxidation, for the potential treatment of obesity and related pathologies, as an alternative or a complement to the classical dietary modification and regular exercise. In Chapter 6, the advantages of rational approaches, over the widely used empirical approaches, to the biotechnological production of phytochemicals of therapeutic interest, based on the knowledge of the molecular and cellular mechanisms that regulate the involved metabolic pathways, are discussed, as well as a particular example of rational approach to the production of the anticancer drug taxol and taxanes. The precise generic delimitation of the genera *Aliella* and *Phagnalon*, their closest relatives within the Gnaphalieae, and the role that hybridization might have played in evolution and diversification of both genera are discussed in Chapter 7. Chapter 8 deals with the assessment of the levels of the mycotoxin patulin in both organic and conventional apples juices and the associated risks for human health. In Chapter 9, the preparation of DNA gel particles using cationic compounds with improved intrinsic biocompatibility, such as amino acid-based surfactants, polysaccharides, and proteins, is reviewed, as well as the role of these cationic compounds in a number of features of these gel particles including DNA load and release. Chapter 10 reports on the evaluation of the effectiveness of combinations of different conventional and non-conventional treatment systems for the reclamation of secondary effluents from urban sewage treatment plants and for obtaining water of sufficient quality for reuse, so that the risks for human health and natural ecosystems are minimized. In Chapter 11 the importance of the occurrence of recombination events and their influence in the genetic structure of *Aeromonas hydrophila* species complex are discussed. Chapter 12 summarizes the entomological and canine leishmaniosis surveys carried out in Northern Spain and Andorra to assess the presence of leishmaniosis or the risk of its transmission by determination of the spatial distribution and density of their vectors.

As in previous editions of this series, this E-book addresses the considerable heterogeneity, multidisciplinarity, and scope of pharmaceutical sciences, through contributions that may be equally valuable in other fields such as biological and chemical sciences.

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1. Supramolecular chemistry for Nanomedicine

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Abstract. Mimicking Nature, supramolecular chemistry represents the chemistry beyond the molecule, in view that intermolecular interactions constitute the driving force for the preparation of molecular and supramolecular assemblies, using the chemical information contained in molecular building blocks. Upon molecular recognition between discrete units, chemical processes such as self-assembly and self-organisation start operating, and are the leading processes to build up supramolecular aggregates and materials. When those materials have dimensions on the nanometric scale, a recently emerging scientific discipline is defined, Nanoscience. Nanomaterials are promising tools for many applications, and their use in biomedical and clinical applications defines the so-called Nanomedicine. In this review we present a few selected examples of nanomaterials designed for therapeutical purposes, emphasizing the importance of the preparation methodology in terms of their therapeutical use.

Introduction

Nanoscience frames the study, manipulation and control of chemical and/or biological materials at the nanoscale, which correspond to structures

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or systems with dimensions within the range of 1 to 100 nm (1 nm = 10^{-9} m) [1]. Within such nanoscale we could include supramolecular biological systems, such as cell membranes, nucleic acids or proteins, as well as supramolecular artificial nanoestructured materials; amongst them, carbon nanotubes, liquid crystals, self-assembled monolayers or supramolecular systems based on colloids, like micelles or liposomes (Fig. 1). Nanoscience is a highly interdisciplinary field as the study of the properties of nanomaterials covers materials science, chemistry, physics and biology.

Two contrasting methodologies to create nanostructures are the so-called "top-down" and the "bottom up" approaches (Fig. 2). In the "top-down" approach, a block of material is taken and carved away until the object that is wanted is reached using techniques such as engraving, photolithography or milling. Thus, the top down approach is based in using nanoengineering and erosion to form the nanomaterial [2].

Instead, in the chemical approach ("bottom up") [3], individual atoms and molecules are driven to or are placed precisely where they are needed by tools such as chemical synthesis, self-assembly or self-organisation. Therefore, supramolecular chemistry and the use of non-covalent interactions is the

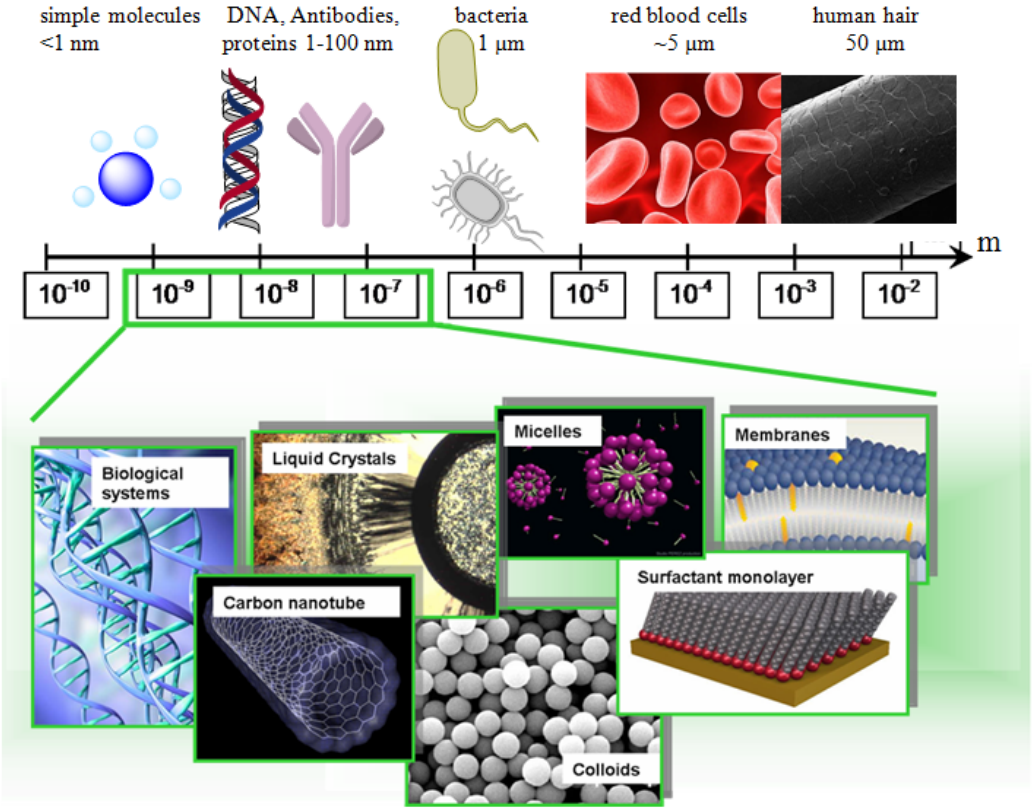

Figure 1. Size scale for different objects, including some soft materials within the nanoscale.

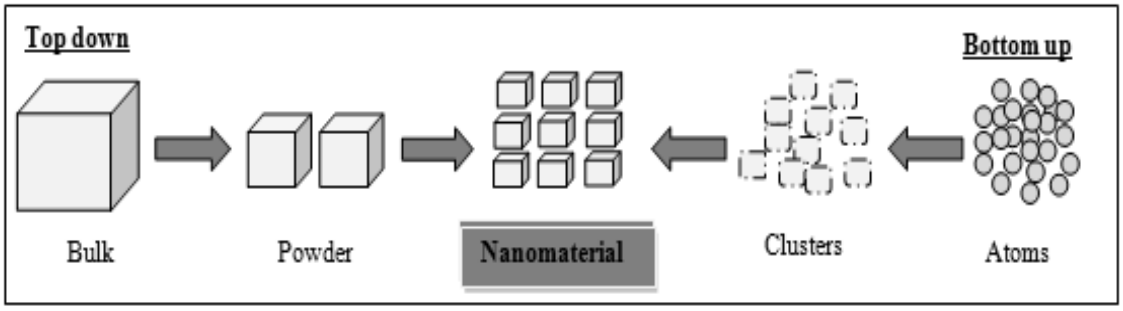

Figure 2. "Top-down" and "bottom up" approaches to the preparation of nanomaterials.

driving force to the formation of nanomaterials [4] by such approach, which relies mainly on the molecular recognition between the molecular components forming the supramolecular aggregates. Examples of this approach expand from the natural world (assembling nucleic acids or molecular motors) to the synthetic one to define the nanochemistry universe (Fig. 3).

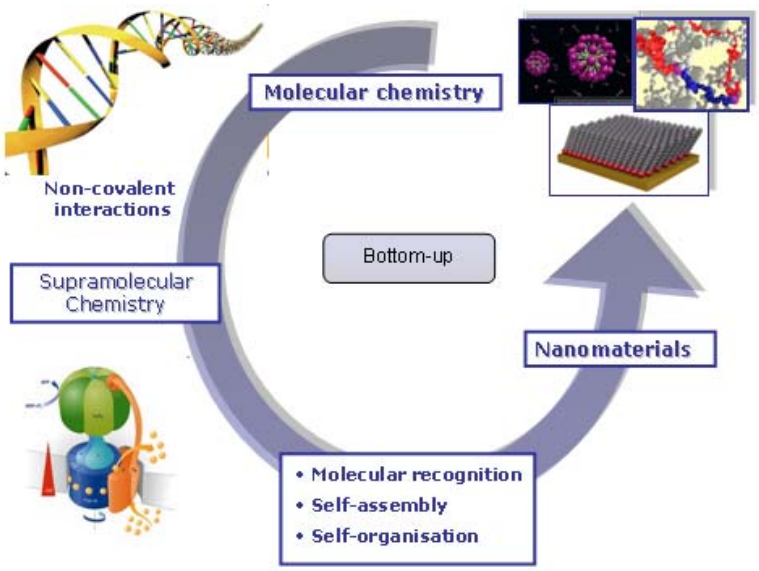

Figure 3. "Bottom up" approach and its driving forces.

On the crossroads between nanotechnology and medicine arises the field of nanomedicine, which is mainly understood as the use of nanotechnological concepts to target medical problems [5]. It is also agreed that nanomedicine revolves around three main areas: medical diagnosis –including imaging-, tissue regeneration and drug delivery (Fig. 4) [6]. In this sense, it could be differentiated from nanobiotechnology because this discipline is more centered on developing basic research to biological systems at the nanoscale [5].

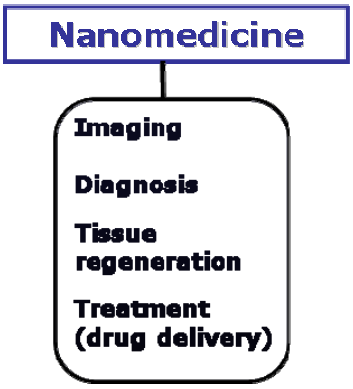

Figure 4. Applications of nanotechnology in medicine: Nanomedicine.

Beyond definitions, it is unarguable that both emerging fields are gaining much attention recently, and they rely on the use of nanostructured materials [7]. Thus, nanomaterials such as nanoparticles, nanofibers, nanowires and nanotubes have novel properties and functionalities which make them attractive to explore and modify biological processes, with potential applications in biomedicine [8]. Some of these nanomaterials are being envisaged and designed as multifunctional nanocarriers, aiming to perform functions as targeting, specific and selective delivering, and sensing [9]. Amongst them, nanoparticles used in drug delivery studies include liposomes, polymers, micelles, quantum dots, gold nanoparticles, paramagnetic nanoparticles and carbon nanotubes, although liposomes and micelles are so far the nanomaterials in clinical use (Fig. 5) [9,10].

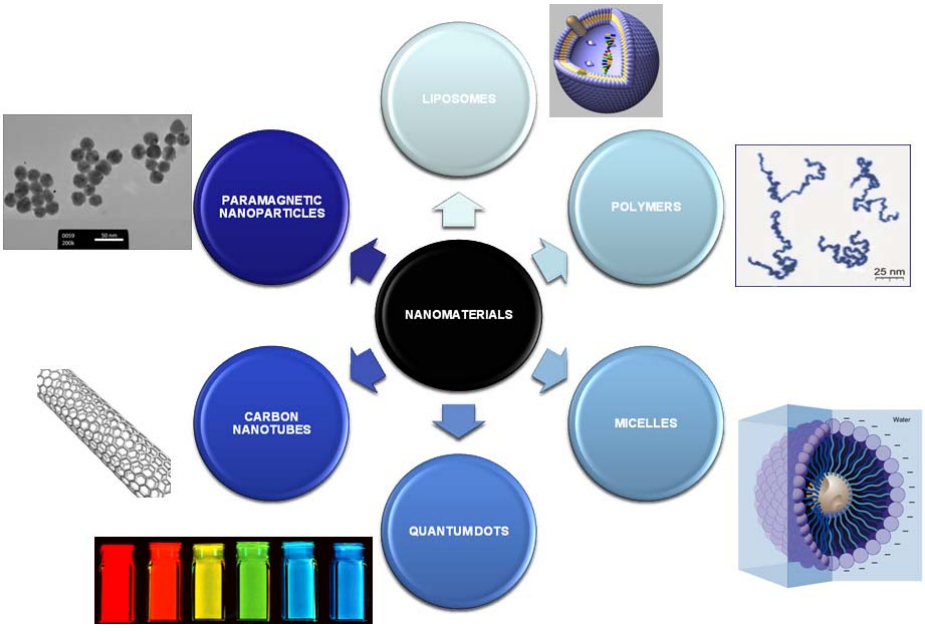

Figure 5. Nanomaterials for Nanomedicine.

In this review, and due to space limitations, a representative selection of examples was needed. Thus, considering the current research activity on this topic, and based in our own research experience, we have chosen some selected examples of the use of these nanomaterials, specifically gold nanoparticles, considered promising tools for both analytical purposes (diagnosis) and therapy (drug delivery). Additionally, we have included a section on self-assembled monolayers, as a representation of how powerful and relevant is surface chemistry for the preparation of functional nanomaterials and devices.

1. Gold nanoparticles in Nanomedicine

The benefits of gold have been known for centuries both in medicine and art (Fig. 6). Its use as medicine has been documented as far as 2500 BC, in China [11]. In the 17th century, one of the most popular ways of obtaining medicinal gold in solution was by dissolving elemental gold in *aqua regia* [12]. However it was not until Faraday's lecture, in 1857, that gold colloid was described as "diffused particles of gold", in a thorough study of its properties, where he described the relationship between the various processes used to obtain gold in different states (including colloidal gold), the sizes of particles obtained, and their relation with the light [13].

Recently, gold nanoparticles (GNPs) have been regarded with interest in the nanomedicine field as agents for labelling and imaging [14], diagnostic or carriers for delivery of biomolecules or small drugs, since they have many features that make them suitable for such applications. One that is of paramount importance is that the gold core is inert, and that GNPs, although

Figure 6. The Lycurgus Cup (British Museum, London) made of dichroic glass, contains colloidal gold and silver that gives its property of being translucent when a light is shone through it.

penetrating the cells, are mainly not cytotoxic. The degree of toxicity depends on the ligand that is stabilizing the gold core [15]. Also, is important the fact that they can be synthesized by simple methods that allow obtaining nanoparticles that are monodisperse, and the surface can be easily functionalized, mainly with thiols but also with others capping agents, such as amines [16], carboxylates [17], or phosphines [18]. There are many reviews focused on the applications of gold nanoparticles [19]. In the present review, we aim at giving a brief overview, with some recent examples found in scientific literature.

1.1. Preparation methods

The most widely used methods for the synthesis of the GNPs are the citrate method, developed by Turkevich, and the Brust-Schiffrin method. In the first one, the gold salt $HAuCl_4$ is reduced by citrate, which also has a stabilizing role [17]. Through this method, one can obtain GNPs that are water soluble, and varying the concentration of the reducing agent it is possible to tune the size of the particles. In the case of the Brust-Schiffrin method, the GNPs are obtained in a biphasic system [20]. In the organic phase there is a thiol, which acts as the capping agent that stabilizes and prevents the growth of the gold atom cluster. In the aqueous phase there is the reducing agent (sodium borohydride) and the gold salt $HAuCl_4$. A phase transfer agent is needed, usually alkyl ammonium halides. The GNPs thus obtained are soluble in the organic phase. Since the surface of the GNP has a monolayer of the thiol attached, sometimes they are referred to as monolayer protected clusters (MPC). In literature, we can also find examples of GNPs that are stabilized by ionic liquids [21] and also gemini-type surfactants [22].

Based in the Brust-Schiffrin biphasic system, our group developed a novel method for obtaining GNPs, using a bis-imidazolium amphiphile of gemini-type synthesized in our laboratory [23] (Fig. 7). This method proved to be suitable for obtaining GNPs that are monodisperse, able to enter cells, with low toxicity, that furthermore could be loaded with a model drug, pursuing its delivery (see below).

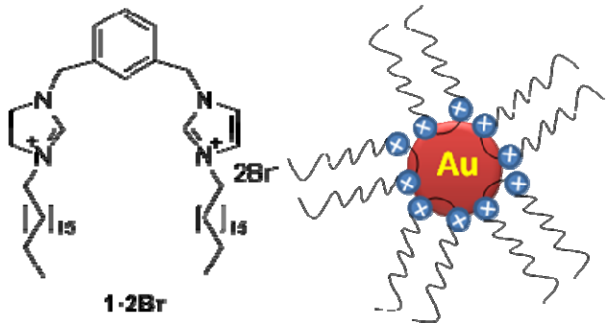

Figure 7. Bis-imidazolium amphiphile 1·2Br (ref. [23]) and its distribution around the gold core of a gold nanoparticle.

1.2. Biomedical applications

GNPs suffer a phenomenon where the free electrons in the surface can absorb electromagnetic radiation, resulting in resonant oscillation. Due to this surface plasmon of the nanoparticles, i.e., propagating electron density waves occurring at the interface between metal and dielectric [14], they can be used as agents for labeling and imaging.

The Surface Plasmon Resonance (SPR) of the GNP can be used for diagnostic in Surface-enhanced Raman Scattering (SERS), which provides noninvasive *in vivo* imaging. The Plasmon resonance of the GNP increases the Raman effect of the molecules in the metal surface, with an increase in the signal by a factor of 10^{14}-10^{15}. Gold nanoparticles were used successfully to detect early-stage inflammatory processes through SERS. They were conjugated with monoclonal antibodies specific for intercellular adhesion molecule-1 (ICAM-1) [24]. The ICAM-1 expression in endothelial cells can be linked to the progression of a wide range of inflammatory, autoimmune and infectious diseases. The GNPs were tested *in vitro* and *in vivo* and showed an S/N ratio that was over 2-fold greater than the one obtained with another technique based in fluorescence microscopy. This approach could also be used in the imaging of cancer cells expressing specific markers. For example, gold nanorods (GNR) were conjugated with a specific antibody and were used to target HER2, a biomarker overexpressed in MCF7 breast cancer cells [25]. Other example used GNP conjugated with antibodies specific for different cancer biomarkers, namely HER2 (overexpressed in breast cancer cells) and EGFR (overexpressed in various cancers). The GNPs showed great stability and good results *in vivo* [26]. The SERS could also be used to monitor the release in the nanomolar range of thiopurine anticancer drugs from the GNP surface (Fig. 8) [27].

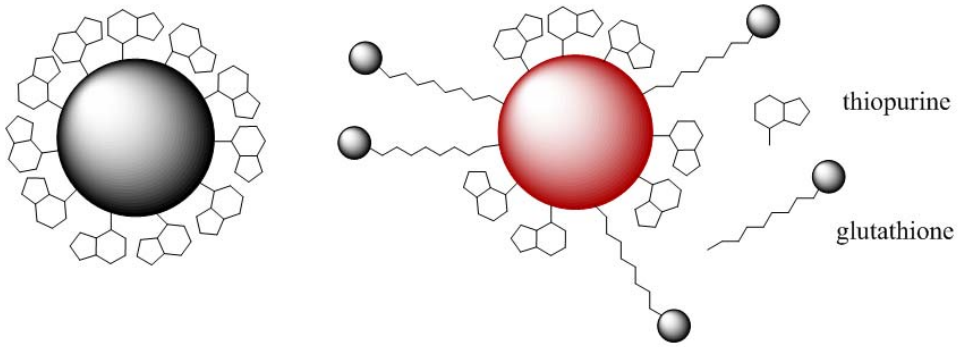

Figure 8. GNP with thiopurine on the surface. Inside the cells, the thiopurine is displaced from the surface by glutathione, it being released into solution, where its concentration can be monitored [27].

GNPs are also being used as contrast agents for Magnetic Resonance Imaging (MRI). In many cases, the nanoparticles have a magnetic core (for example, Fe_3O_4) and a shell of Au that allows further functionalization with the desired molecule on the surface. This type of nanoparticles was studied for the diagnosis and therapy of prostate cancer [28]. The nanoparticles were conjugated with a monoclonal antibody specific for the prostate stem cell antigen that prostate cancer cells overexpress. The monoclonal antibody (mAb) targets the cancer cells, but it is also used in immunotherapy. MRI was used to assess *in vivo* biodistribution of the nanoparticles-mAb conjugate. Manganese-gold nanoparticles are other example that is being studied. Mn^{2+} can be used as positive aqueous-based contrast agent for MRI imaging. These multifunctional Mn-GNPs were tested *in vitro* and *ex vivo*, and it was observed that they had low cell mortality and a high signal suitable for application *in vivo* [29]. Due to the versatility of these materials, alternatives are studied where the nanoparticles obtained can be used for both MRI and SERS [30]. In this case, the GNPs are complexed with iron oxide nanoparticles coated with dextran (Fig. 9). The dextran-iron oxide particles have already been studied as contrast agents for MRI, and the gold is deposited on its surface and functionalized with a reporter molecule that is used for detection through SERS.

Besides its use in imaging, GNPs can also be used as therapy agents, both alone, in photothermal therapy, or as drug delivery agents. In the first case, the photothermal therapy is based on the SPR of the GNP: when nanoparticles are irradiated with the adequate energy, they convert the light into heat that is responsible for the increase in the temperature, resulting in the ablation of the tumor [31]. Although the nanoparticles tend to accumulate in tumors (due to their high vascularization), the accumulation can be enhanced through the conjugation with ligands specific for the receptors that

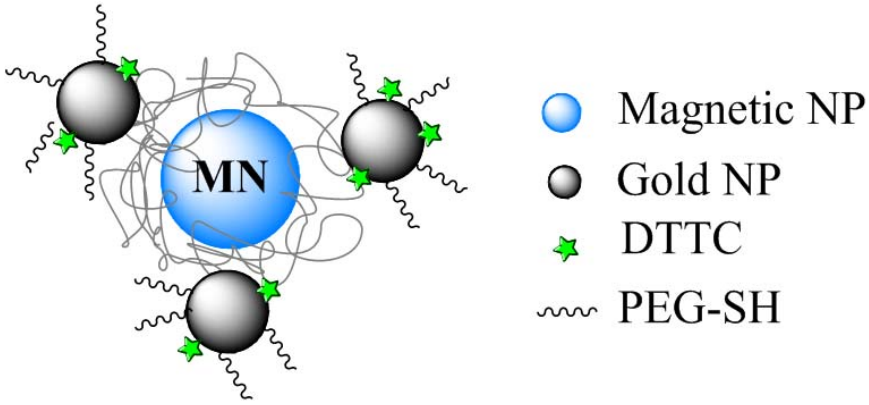

Figure 9. Iron oxide magnetic nanoparticles (MN) are covered with dextran. Gold nanoparticles (GNPs) are grown on its surface, and a thiol with PEG and a reporter molecule (DTTC) are attached [30].

are overexpressed on the tumor cells. One example found in literature is the use of ephrinA1-conjugated nanoshells. This ligand is specific for the EphA2 receptor that can be found in many prostate cancer cell lines. This approach was successfully used to target and destroy PC-3 prostate cancer cells *in vivo*, when a NIR laser was applied [32]. Similarly, colorectal tumors that overexpress guanylyl cyclase C (GCC) have been targeted through the conjugation of gold nanoshells with a heat-stable enterotoxin which is recognized by the receptor [33].

The work we developed in our group with imidazolium based gold nanoparticles included the study of their potential as a drug delivery system [23]. In this particular case, the synthesized gold nanoparticles (Fig. 10) were successfully used to incorporate an anionic model drug (ibuprofenate) and deliver it in a sustained manner. Furthermore, the nanoparticles presented relatively low toxicity, and could be internalized by cells.

GNPs are also widely studied to be used as drug vectors. For example, some amino-glycosidic antibiotics (streptomycin, neomycin, gentamycin and kanamycin) were conjugated with BSA capped GNPs, and presented *in vitro* better antibacterial activity than the antibiotics alone [34]. In a similar way, the conjugation of oxaliplatin to GNPs showed an enhancement in the delivery of the drug to lung cancer cell line A549 and colon cancer cell lines HCT116, HCT15, HT29 and RKO, besides an increase in the penetration in

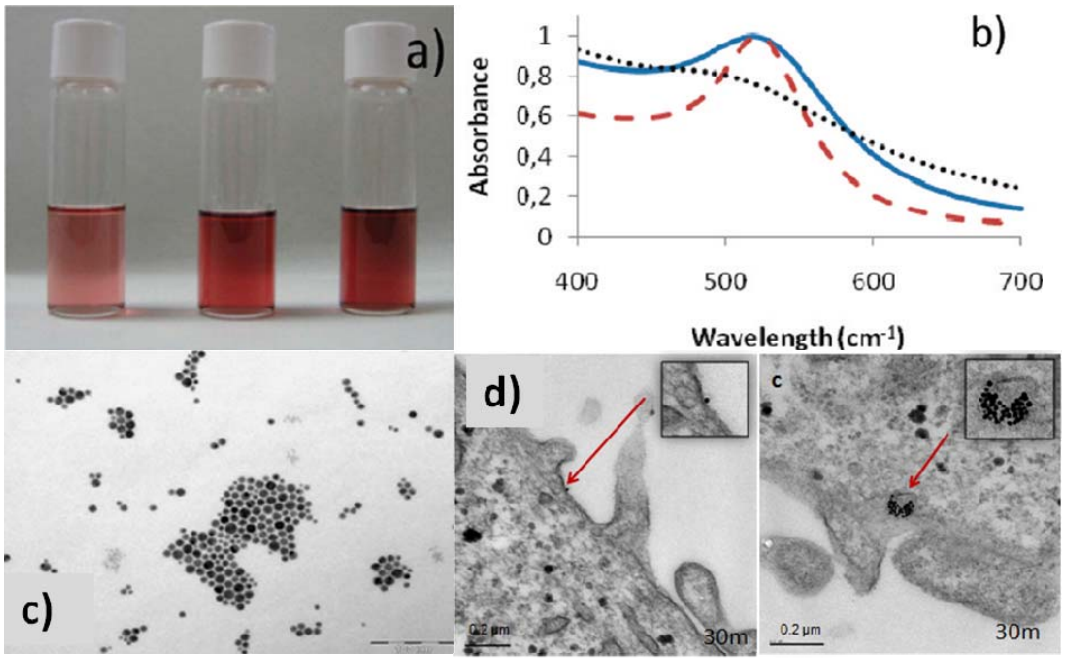

Figure 10. a) Images of gemini-imidazolium stabilized gold nanoparticles in dichloromethane, and images of their b) UV-Vis absorption spectra; c) TEM micrograph, and d) TEM images of nanoparticles internalized in Caco-2 cells [23].

the nucleus of the A549 cell line [35]. The enhancement can also be achieved through targeting, in the same way as described above for the GNPs used in imaging. For example, GNPs that were conjugated with curcumin, a potent anticancer molecule, and hyaluronic acid, which has strong affinity for cell surface markers CD44 and RHAMM. The markers are overexpressed in many types of tumors, probably because of the relation between hyaluronic acid and the angiogenesis. Furthermore, the GNPs were conjugated also with folate-PEG, to enhance their targeting ability, which resulted in the desired intracellular accumulation of GNPs [36]. The same folate ligand was used in GNPs conjugated with doxorubicin for targeting and delivery to tumor cells. Testing in A549 (non-expressing folate receptor) and KB (expressing folate receptor) cell lines showed a doxorubicin intake higher than for the drug alone in both cell lines, but the uptake was higher in KB cell line due to the folate receptor [37]. Similarly, GNPs synthesized with a copolymer of aspartate-doxorubicin and PEG, and conjugated with folate showed a drug release made in a sustained manner, depending on the pH of the medium [38]; the release is faster in acidic medium due to the hydrolysis of the hydrazone linkage. GNPs functionalized with a thiol-PEG derivative of tamoxifen, which competes with 17β-estradiol for the estrogen receptor alfa (ERα), could be delivered selectively to breast cancer cells expressing ER, meaning that the receptor may aid the selectivity and uptake. The potency of the drug action in these breast cancer cells was up to 2.7 times higher [39]. GNPs covered with cyclodextrin, that provides a pocket to encapsulate β-lapachone, an anticancer drug, and functionalized also with anti-EGFR antibody, for targeting, were developed. In this case, the release of cyclodextrin from the GNP is induced by the presence of glutathione, followed by the release of the drug from the cyclodextrin pocket [40]. In this case, the GNP has a targeting function besides the triggered release of the payload.

1.3. Perspectives

Due to its versatility, GNPs are one of the nanostructured materials more widely studied, and has found application in distinct areas. Besides the given examples, the GNPs are also popular for use in diagnostic test kits. As outlined before, the examples shown here are just a small description. In the data below, one can see that the number of publications related with gold nanoparticles has seen an exponential increase (Fig. 11).

We expect to see very soon some of these systems clinically available, since they are being subject of active research in many fields, and show such promising results.

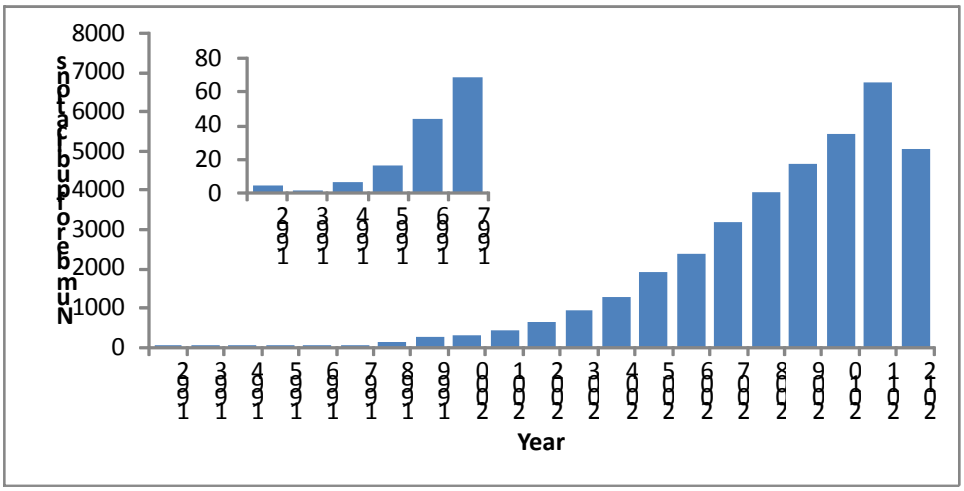

Figure 11. Number of publications with gold nanoparticles per year [41].

2. Self-assembled monolayers as (bio) sensors

Covalent immobilization of biomolecules or synthetic bioactive molecules onto solid supports is a key step in the area of biosensors, biotechnology and nanosciences [42]. One of the most currently used methods to immobilize biomolecules to substrates is the formation of an organic monolayer that will act as linker, the so-called self-assembled monolayer (SAM). SAMs are molecular assemblies formed spontaneously on metallic or inorganic surfaces by chemisorption between the substrate and a functional head group [43]. SAMs provide one simple route to functionalize surfaces by organic or biologic molecules containing free anchor groups such as thiols, disulfides, amines, silanes, or acids. The monolayer produced by self-assembly allows high flexibility with respect to several applications depending upon their terminal functionality or by varying the chain length (distance control). SAMs are also used as model substrates in biological studies because of their well-defined structure, controlled surface properties and biocompatibility. SAM formation allows the possibility of changing surface properties or to add new characteristics to the materials. The self-assembling molecules suitable to generate functional SAMs consist generally of three parts: the head group, the alkyl chain and the terminal end group (Fig. 12).

The head group is responsible for the anchoring of the molecules onto the substrate. The alkyl chain has a significant influence on the ordering of the SAM and provides the stability of the monolayer, due to van der Waals interactions. The terminal end group (functional group) introduces chemical functionality into the monolayer system and is important for the overall properties of surfaces because it allows the immobilization of the desired active molecule [44]. The control of chemical and physical properties of the

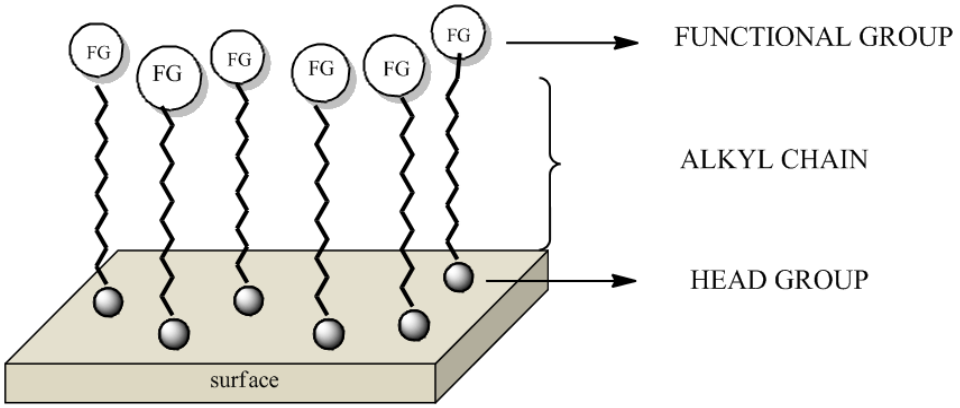

Figure 12. Fragments of functional SAMs.

SAMs has recently gained considerable attention, since, by selecting precursor molecules with adequate terminal functional groups, the functionalities of these surfaces can be regulated at the nanoscale.

2.1. SAMs formation and characterization

Different materials and assembling molecules have been employed as substrates for the formation of monolayers, but gold and silicon are the most widely used and studied because of the ease of using thiol and silane chains, respectively, to be attached onto the corresponding surfaces; they form well organized SAMs and they are biocompatible materials [45]. For example, SAM of long chain alkane thiol produces a highly packed and ordered surface, which can provide a membrane like microenvironment, useful for immobilizing biological molecules.

SAMs are formed spontaneously and can be produced usually through physical vapor deposition technique or by immersing a substrate into a solution of the molecule of interest (wet chemistry) [46]. SAM formation is a chemical process that depends on several parameters such as: deposition time, type of substrate and assembling molecule, type of solvent and deposition methodology. For example, to form a typical SAM on a gold surface, a diluted solution of the thiol in ethanol can be completed in less than 1 h, although the SAM will be more ordered if the deposition time increases. Fig. 13 shows a schematic representation of the thiol formation on a gold surface.

The best option to have a good characterization of a SAM relies on the combination of several analytical techniques, which is essential in order to confirm the SAM formation and ensure its functionality. One of the most used techniques is Atomic Force Microscopy (AFM), a very high-resolution

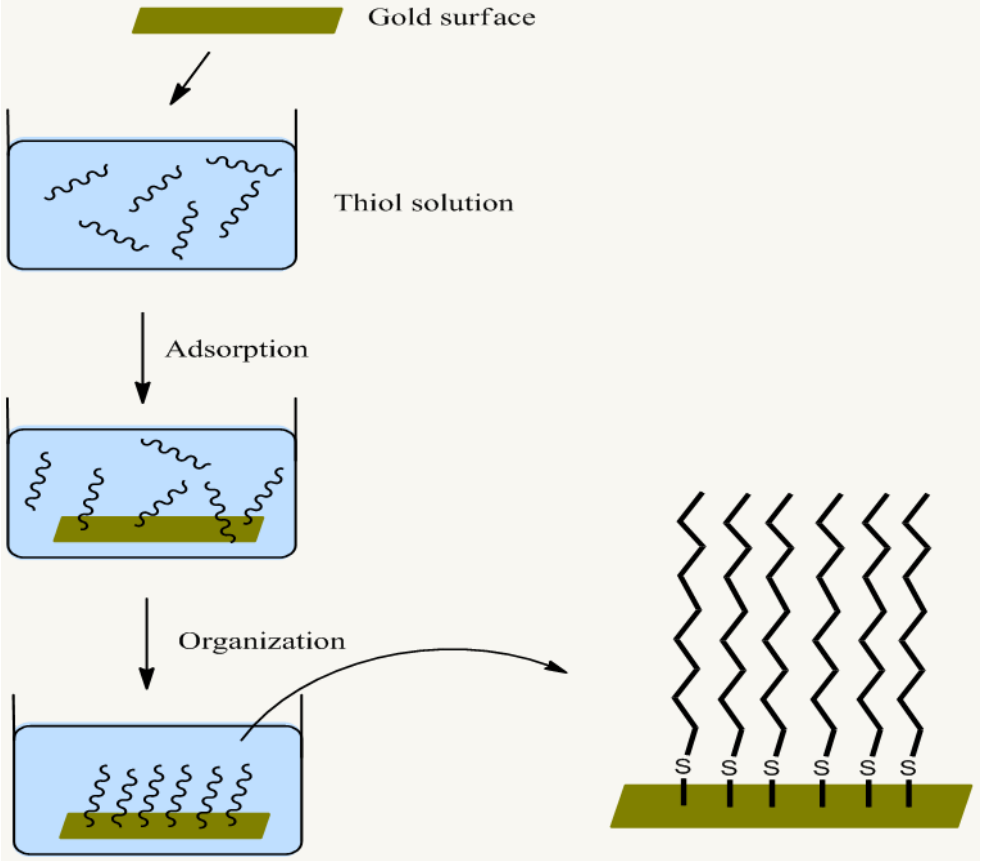

Figure 13. Schematic representation of SAM formation on gold.

type of scanning probe microscopy that allows the study of the surface coverage [47]. The information is obtained by "feeling" the surface with a piezoelectric (the cantilever), that explores the sample at the nanoscale with accurate and precise movements guided by a laser light from a solid state diode.

On the other hand, mass spectrometry techniques can be also used, such as matrix assisted laser desorption/ionization (MALDI-ToF MS), allowing the analysis of biomolecules and large organic molecules attached on the surface. MALDI MS has been used for SAM characterization first by Mrkisch, and it is used specially for gold surfaces because the fragmentation is lower and the total mass of the thiol can be detected [48]. Time of flight secondary ion mass spectrometry (Tof-SIMS MS) is a technique used to analyze the composition of solid surfaces, it being able to detect chemical elements or biological fragments such as amino acids [49]. Additionally, X-ray photoelectron spectroscopy (XPS) is a surface chemical analysis technique that allows the quantitative analysis of a material. XPS can measure the elemental composition, empirical formula and the chemical and electronic state of the elements present in a material [50].

Contact angle measurements are simple to make, and offer useful information about the hydrophobicity of a surface, and the changes that occur through surface modification. The method consists of depositing a drop of water on the functionalized surface and to record the angle formed between the drop and the surface [51]. Depending on the characteristics and the terminal group of the SAM, the hydrophobicity of the surface can be assessed. Fig. 14 shows an example of polysilicon functionalization methodology and the contact angle values associated to each chemical step. The initial polysilicon surface is quite hydrophobic (θ = 70º), but after hydroxylation the surface becomes highly hydrophilic (θ = 25º). Finally, when a SAM was formed using 11-triethoxysilaneundecanal (TESUD), the contact angle value increases noticeably, indicating the presence of the hydrophobic aldehyde SAM (θ = 98º) as well as a high level of surface functionalization.

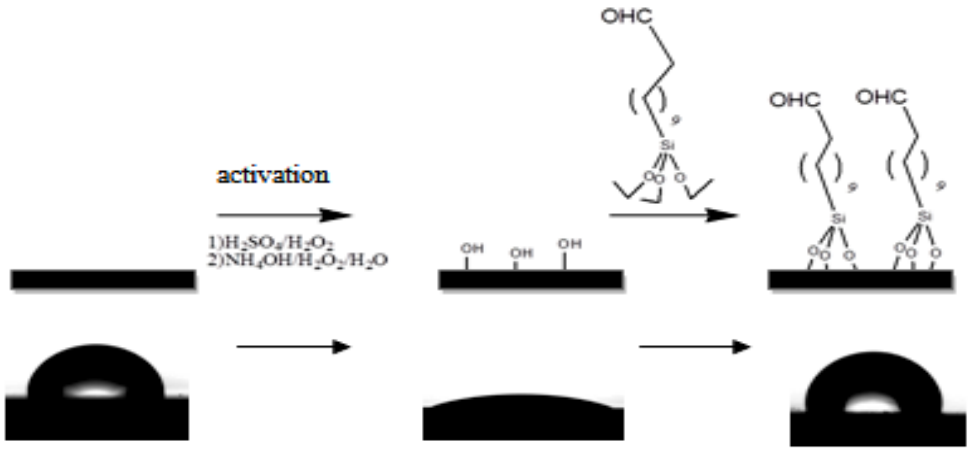

Figure 14. Contact angle measurements of clean polysilicon (θ = 70º), hydroxylated surface (θ = 25º) and TESUD monolayer (θ = 98º).

2.2. Biomedical applications of SAMs

SAMs have been studied and investigated in the last decade for their high range of applications in many areas such as nanotechnology, chemical surface sciences, biotechnology, chemical engineering or electronics. The activity and the utility of the SAM depend on the molecule that will be immobilized into the SAM using the reactivity between the functional group of the SAM and the desired molecule [52].

Biofunctionalization of SAMs with different biomolecules is of great interest to give biological properties to the substrate, and the preparation of biosensors is one of the main applications of the biofunctionalized SAMs

[53]. The selectivity offered by biomolecules such as antibodies, proteins, nucleic acids and enzymes or even organised systems like whole cells can be profitably used for molecular recognition. As a consequence, immobilization strategies for biomolecules are of paramount importance in order to preserve their biological activity. Various types of immobilization procedures are able to link the biomolecule with the substrate. These procedures could be classified according to the covalent and non-covalent natures of the linkage [54], although the covalent bond is more used due to their strength and stability.

Proteins and other biomolecules can be covalently linked to SAMs via the *N*-terminal amino group [55]. Primary amines react with activated esters by nucleophilic attack resulting in the formation of an amide bond. As it has been reported in the literature, two classical ester activating groups, the tetrafluorophenyl ester (PFP) [56] and the *N*-hydroxysuccinimide (NHS) [57] can react readily with amine containing biomolecules to obtain an amide bound (Fig. 15a). Also, the aldehyde group can be used to attach biomolecules covalently to gold or silicon surfaces [58]. In this case, the amine groups in the biomolecule react with the aldehyde groups in the SAM to generate a stable secondary amine linkage by reduction of the intermediate imine (Fig. 15b).

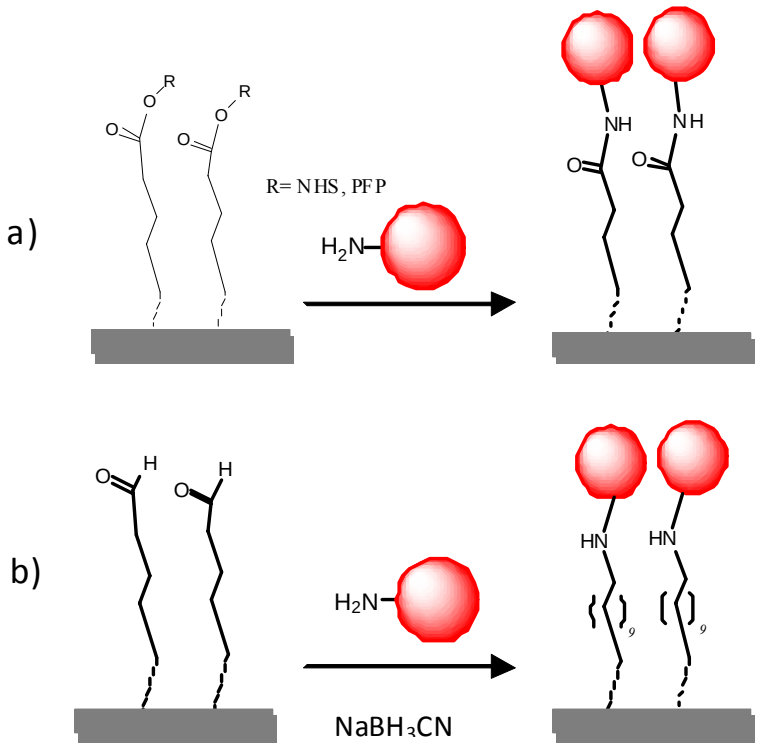

Figure 15. Protein immobilization through a) amide bond and b) amine linkage.

Examples of diagnosis systems based on SAMs are the personal glucose test devices or the pregnancy test kits [59]. Several biosensors also exist, of interest in Nanomedicine, based on the immobilization of antibodies or proteins that can recognize specific analytes. Fig. 16 shows an example of a protocol for the SAM formation on gold surface, followed by antibody immobilization and analyte testing for cancer detection [60]. First, a mixed SAM was structured using a) a terminal carboxylic acid thiol, which will react with the biomolecule, and b) a hydroxyl terminal thiol. The mixed thiols were chosen to provide appropriate organization and stability to the SAM, and to give the sufficient space to allow the active group to link the antibody. Next, a classical acid activation was done using NHS and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) to connect the antibody covalently to the SAM. The immobilized antibody recognizes a specific antigen, thus acting as a nanobiosensor.

On the other hand, organic molecules can be also immobilized through a SAM. Normally, fluorescence moieties are included in the system, in order to detect changes depending on the parameter that has been studied [61]. The importance of sensing many biological processes has led to the development of fluorescence based organic receptors which can be used as chemosensors exhibiting a variation on their intrinsic fluorescence. The concept of organic receptor immobilization on functionalized substrates relies on detecting fluorescence changes in the presence of the given analyte (Fig. 17).

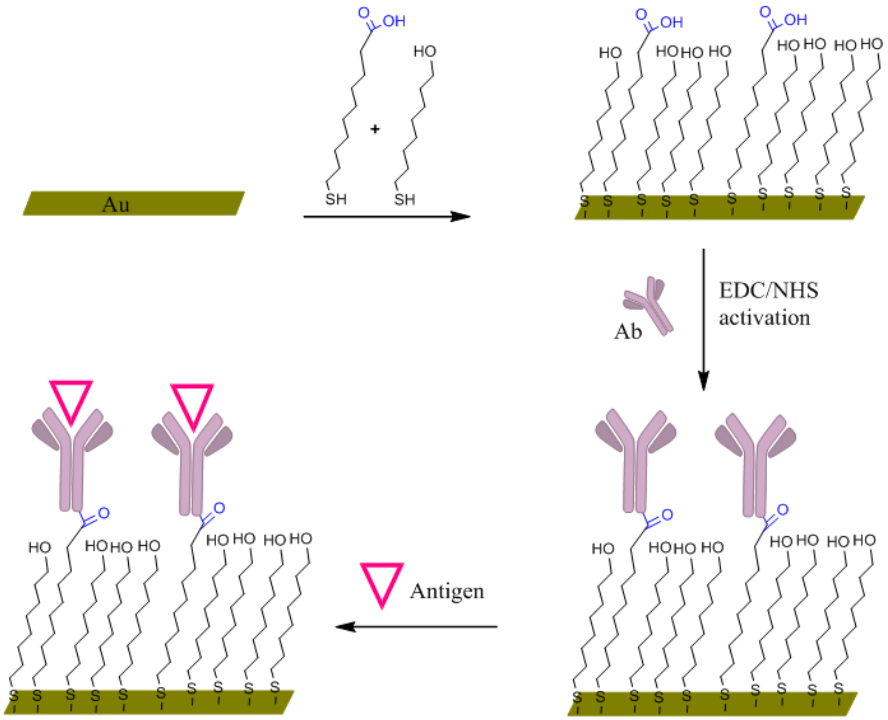

Figure 16. Antibody functionalization example.

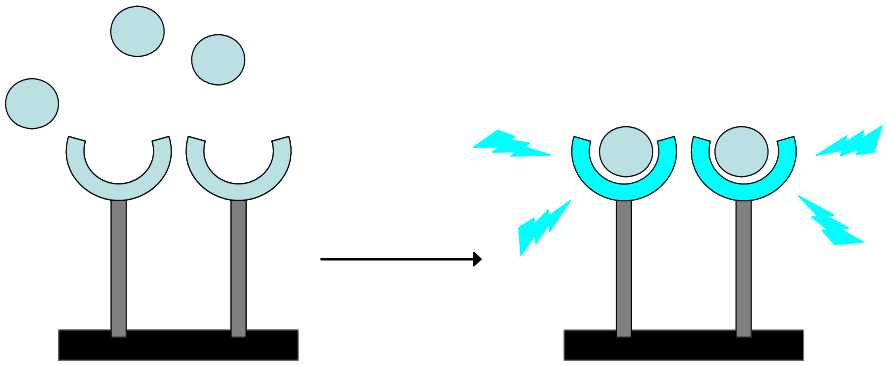

Figure 17. Model of fluorescence chemosensor.

Supramolecular chemosensors are composed of a binding part for guest recognition, and a sensing moiety to read information about the complexation.

Immobilized chemosensors have been studied in the last years for sensing important biological parameters at cellular level such as biological ions or radicals as well as enzyme activity, using fluorescence changes because it is one of the preferred methods for detection in living organisms [62]. As example of chemosensor, one of the biological parameters that has been studied in the last years is the level of various reactive oxygen species (ROS), because they are implicated in many intracellular processes going from apoptosis or cellular activity to a variety of inflammatory responses [63]. Fig. 18 shows a model of ROS sensor based on anthracene moieties [64].

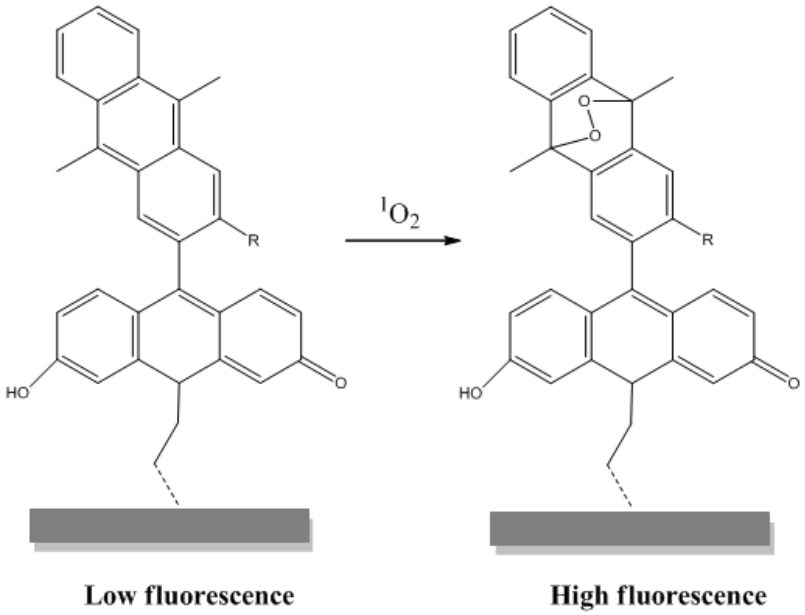

Figure 18. ROS formation induces change on the fluorescence emission of the immobilized anthracene containing derivative.

The presence of singlet oxygen detecting ($^1O_2$) produces the formation of an endoperoxide species, and the fluorescence of the complex increases. The system is hydrophilic, which is important for its use in biological samples, and furthermore, it is very selective for detecting $^1O_2$. Therefore, appropriated SAMs can be used as fluorescent probes for recognizing specific analytes in a selective manner, with potential usefulness as sensors for biological systems.

SAMs were also used recently by us pursuing cell tracking, a promising approach to individual living cells identification. Silicon barcodes were designed for extracellular tagging using photolithography processes [65] and were modified using chemical biofunctionalization. A self-assembled monolayer was used as a connector between the silicon material and the lectin wheat germ aglutinin (WGA) [66]. WGA was used because of its capacity to recognize some specific carbohydrates present on the surface of most mammalian cells. SAMs were prepared on polysilicon surfaces including aldehyde groups as terminal functions to study the suitability of their covalent chemical bonding to WGA. Fig. 19 shows an outline of the biofunctionalized barcodes that were tested doing adhesion experiments to the zona pellucida of mouse embryos. These experiments showed high barcode retention rates after 96 h of culture as well as high viability indicating the robustness of the biofunctionalization.

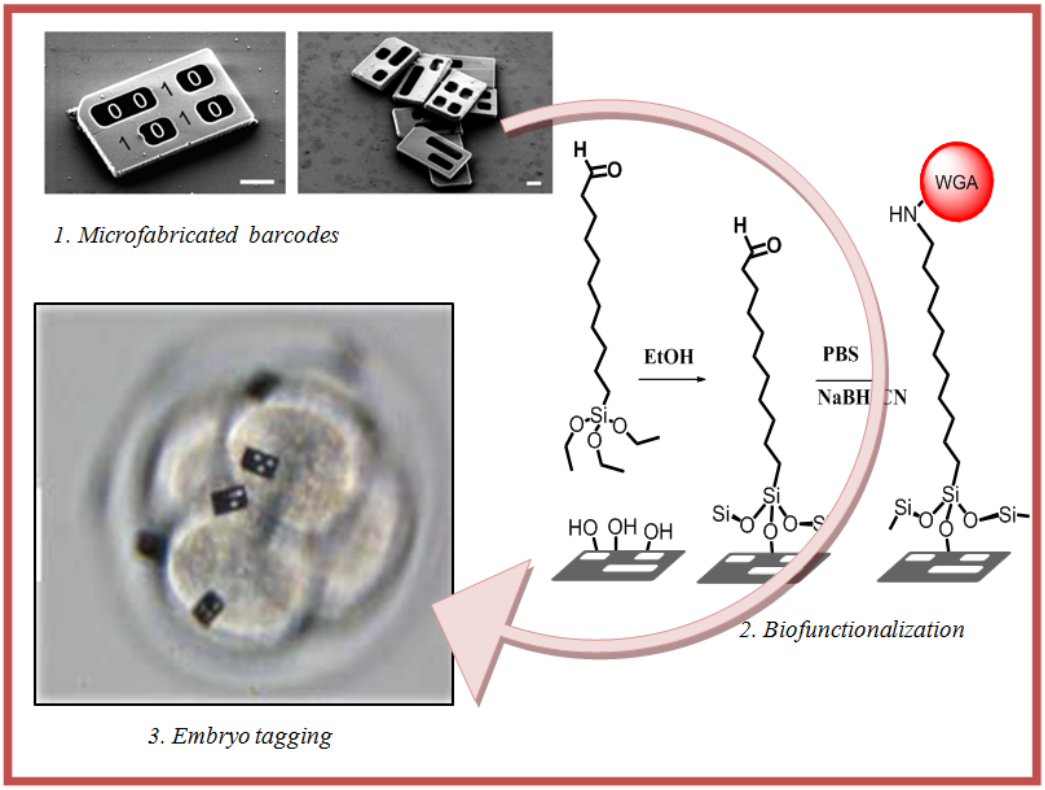

Figure 19. Representation of WGA biofunctionalized barcodes for embryo tagging.

3. Conclusion

We have shown various examples of nanomaterials of interest in Nanomedicine, mainly nanoparticles and self-assembled monolayers, some developed by our research group. The biofunctionality of any of the described nanomaterials is determined by a combination of an accurate design and selection of the components of the systems, as well as a shear control of the self-assembly processes governing their synthesis. Supramolecular chemistry lies behind any of these processes and it is central to any successful approach leading to new and promising nanomaterials for healthcare.

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2. Antifungal compounds from plants

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Abstract. Due to the increase of the incidence of fungal infections in humans and the limitations of the available antimycotic drugs, among which the emergence of resistant strains, there is a need for the discovery of new antifungal agents. Plants, especially those used in Traditional Medicine, linked to an unmatched chemical diversity, either as pure compounds or as plant extracts, provide unlimited opportunities for the development of new antifungals. In recent years, compounds from different phytochemical groups have been described as having antifungal activity, including polyphenols, saponins, or peptides, among others, as well as essential oils and their constituents. After screening of ethnopharmacologically selected plants, mainly from Latin-America, followed by bio-guided isolation, our group has described the antifungal activity of different types of plant constituents, such as sesquiterpenes, triterpenes, flavonoids, lignans, fatty acids and essential oils.

Introduction

Fungal infections arise as an increasing health problem with a high economic cost. A recent study of the epidemiology of sepsis caused by fungi in USA showed that between 1979 and 2000 they increased by 207%.

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The morbidity and mortality associated with these infections are substantial: deaths due to mycoses increased from 1557 in 1980 to 6534 in 1997, in the USA, and they were mainly associated with *Candida*, *Aspergillus* and *Cryptococcus* sp. infections [1-3].

The most frequent fungal pathogens include the yeasts *Candida* sp., (mainly *C. albicans* and other species especially virulent, like *C. glabrata*) and *Cryptococcus* sp., filamentous fungi like *Aspergillus* sp., *Fusarium* sp. and *Rhizopus* sp., and the dermatophytes *Trycophyton* sp., *Microsporum* sp. and *Epidermophyton* sp. Particularly, *Candida* and *Aspergillus* represent 70-90% and 10-20%, respectively, of all invasive fungal infections [4].

A number of reasons have led to the significant augmentation of the incidence of fungal infections in humans in recent decades [1,4,5]:

- The increase in the number of immunocompromised patients who frequently develop opportunistic superficial and systemic fungal infections.
- The invasive medical procedures in hospitals (use of catheters, peritoneal dialysis, haemodialysis, parenteral nutrition, etc.).
- The emergence of new and uncommon virulent fungal strains, especially favoured by an increase of the population mobility that facilitates a higher exposure to endemic fungal pathogens.

The evolution of fungal infections is further worsened by the fact that they are often difficult to diagnose, being recognized when they are already at an advanced stage.

Currently, there is a variety of antifungal drugs with different mechanisms of action. However, their effectiveness is limited due to a number of factors, such as fungistatic mode of action, lack of oral and intravenous preparations due to low solubility, drug toxicity and the development of drug resistance [6,7].

From another standpoint, fungi cause significant losses in agronomy, taking in account that, depending on the crop type and the region, approximately 10-20% of the cultivated food plants are destroyed by plant pathogens, among them pathogenic fungi. The use of fungicides is still considered essential for increasing crop yields [8].

1. Search for new antifungals

So, therefore, there is a clear need to search for new antifungal agents providing new mechanisms of action, with a broad spectrum of antifungal activity, fewer dose-limiting side effects, and economic [9,10].

Nature offers a wide chemical diversity and natural products are an important source for the development of new therapeutic agents, in particular anti-infective agents. Some of the antifungal drugs most recently introduced in clinical practice (echinocandines and sordarines) are derived from natural products [4,11].

Among the different natural sources of active principles, the plant kingdom has a special significance:

- Plants offer a wide biodiversity that also involves a high chemodiversity.
- It is estimated that less than 10% of the plant secondary metabolites have been isolated. In many cases these substances act as plant's defence mechanisms against attack by microorganisms, insects, etc. [12].
- Plants are an important source of medicinal preparations (extracts, teas, essential oils, etc.) used in traditional medicine since antiquity, and particularly many of them in the treatment of infectious diseases. It is estimated that 14-28% of species of higher plants are used in therapy.

In addition, to date, more than 600 herbal drugs showed antifungal activity, but only a small part of them have been studied concerning their active constituents.

Therefore, either as a source of pure compounds or extracts, the plant kingdom offers a huge potential for new drug development.

2. Methods for research of antifungal agents from plants

2.1. Selection of plant species

The first key point is the selection of plant species to be studied, from which the success of the research depends greatly.

The ethnopharmacological selection is based on existing knowledge about the traditional use of plants to treat diseases and it has shown to increase the probability of success in drug discovery, reducing empiricism.

The isolation and characterization of bioactive molecules from ethnopharmacologically selected plant species allows not only their use as drug or as chemical or pharmacological leads to produce new analogues, but also, and not less important, to set a scientific basis for the development and use of herbal drug preparations and to validate the use of traditional medicinal preparations.

Therefore, there is a strong interest in performing screenings of antifungal activity from medicinal plants used in traditional medicine for the treatment of fungal diseases [13-24].

The first step in the ethnopharmacological-based research is the collection of data (most used plant species, part of the plant, forms of preparation and administration, uses, etc.) from indigenous population and their analysis. It is essential that the collected plant material is correctly authenticated by a botanist and that a voucher specimen is included in an internationally recognized herbarium.

Most of the research conducted by our group has been performed on Latin American plants. Latin America is a very suitable source of plant material for drug discovery because [14]:

- It has a high biodiversity, one of the highest in the world in terms of vascular plants.
- Many of the plant species have never been investigated for the research of bioactive agents.
- There is a rich tradition in the use of medicinal plants and this ethnopharmacological knowledge has been relatively well preserved by the indigenous population.

2.2. Preparation of crude extracts

Both, the appropriate extraction method and the most suitable solvents should be carefully selected. Concerning the method, usually plant extracts are obtained by maceration or percolation. Since the chemical nature of the active principles to be isolated is not known, the plant material is often subjected to a successive extraction with increasing polarity solvents (for example, Cl_2CH_2 and MeOH 95%) to obtain a wide range of compounds of different polarities. Additionally, an extract equivalent to the preparation commonly used in traditional medicine (usually aqueous or alcoholic extract) should be prepared. In the particular case of aromatic herbs, it is useful to obtain the essential oil by hydrodistillation.

2.3. Screening of the antifungal activity

For the screening of antifungal activity and its monitoring during the isolation and purification of the active principles, test methods based on diffusion are mostly used, whereas to quantify the activity, both, dilution and diffusion methods may be employed. Reviews on the different techniques used for the evaluation of the antifungal activity of natural products can be found in Cos *et al.* [25], Das *et al.* [26], Engelmeier and Hadacek [8], Jacob and Walker [27] and Ríos *et al.* [28].

One of the most widely employed methods to assess the antifungal activity of plant extracts is the agar disk diffusion assay [29].

In our research, a number of plant extracts and essential oils, mainly coming from plants traditionally used for the treatment of skin diseases, were subjected to a screening of antifungal activity using the agar disk diffusion assay against a panel of fungal strains including both yeasts and filamentous fungi. Among them, there were opportunistic and skin and mucosal membranes pathogenic fungi, as well as phytopathogenic strains that can cause significant losses in agriculture and hinder food conservation [15,16]. The most active extracts were selected for subsequent bio-guided fractionation with the aim of isolating and characterizing the active principles.

2.4. Bio-guided isolation and identification of active constituents

The most time-consuming step is the isolation and purification of the active compounds, which is mainly achieved through bio-guided fractionation. It is based on the combined use of preparative chromatographic techniques (e.g. column chromatography, medium- and high-pressure liquid chromatography, classical and centrifugal thin-layer chromatography, counter current chromatographic techniques) [30] together with bioautography with the aim of selecting in each step of the chromatographic fractionation the fractions containing the active compounds for further purification.

Contact bioautography allows the detection of active constituents in a complex mixture within a reasonable time. It is an agar diffusion method in which the components of a sample (raw extract or fraction) previously separated by thin-layer chromatography (TLC) diffuse from the stationary phase into the culture medium inoculated with the fungus that has been more sensitive to the crude extract in the antifungigram [31].

To do this, the already developed and dried TLC plate is placed overlaid on the inoculated Sabouraud dextrose agar medium, leaving enough time (1 h at 4ºC) so that diffusion takes place. Then, the TLC plate is removed and the inoculated agar incubated at 30ºC for 48 h. The growth inhibition zones observed are related to the substances separated on a TLC reference plate developed with the same mobile phase. In this way, active compounds can be detected on the TLC plate and the fractionation can be directed to the isolation of these compounds.

Some considerations should be taken into account when evaluating the results of a bioautography: a) due to the limited exposure time (usually 1 h) of the inoculum to the potential antifungal agents the sensitivity is lower than in the antifungigram, b) according to their polarity the components of the sample will diffuse differently through the agar, and c) the result may be affected by the stability of the product on the TLC plate [27].

Nonetheless, bioautography is a useful technique which complements the antifungigram, since it allows the detection of active substances that in the initial raw extract are present in a very low concentration, or that coexist with others that antagonize their activity.

Furthermore, it is necessary to bear in mind that during the fractionation of an active extract a loss of activity may occur due to a degradation of active principles during isolation, or to the separation of different components of the extract acting synergistically.

Finally, once the active compounds are purified, their chemical structure should be established by using different standard spectroscopic techniques, such as UV-Vis, IR, different types of mass spectrometry, and a variety of experiments of ^{1}H-NMR and ^{13}C-NMR, involving both mono- and bidimensional methods.

Particularly, in the case of essential oils, identification of the constituents of the oil and their fractions is usually achieved by means of qualitative and quantitative analysis by GC-FID, GC-MS [32] and also, if necessary, by ^{13}C-NMR [33-35].

2.5. Evaluation of the antifungal activity

The antifungal activity of the extracts, essential oils and pure isolated compounds should be quantitatively evaluated through determination of their minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC).

The MIC is defined as the minimal concentration of a product that inhibits the visible growth of microorganisms. It allows quantification of the sensitivity of a certain fungal strain to an antifungal agent. MIC is usually determined using either broth macrodilution or microdilution methods, the latter being the most used, through a standard two-fold dilution technique [36]. In the case of lipophilic substances that are dissolved in solvents poorly soluble in water, addition of Tween 80 as an emulsifier is recommended.

Because samples may not be 100% antifungal at their MIC, viable fungal cells may remain in the wells in which fungal growth is not appreciated by sight. Thus, the determination of the MFC is also necessary to establish their fungicidal activity. The MFC can be established technically by extending the MIC (microdilution method) [37].

3. Major chemical groups with antifungal activity

A number of compounds isolated from plants are reported to have antifungal activities. However, development of useful antifungal drugs from

the majority of them has not yet been possible, even though mainstream medicine is increasingly receptive to the use of antimicrobial and other drugs derived from plants, because of the resistances developed against traditional antibiotics [12].

One of the reasons is the fact that most of the antifungal plant products, i.e. extracts, essential oils or pure constituents, have only been tested for their *in vitro* activities, their effectiveness in animals and/or humans remaining unknown. Pharmacokinetics, undesirable effects, interactions and toxicity should also be studied [38].

The interest of finding new antifungal compounds from plants is that they can constitute precursors leading to more active antifungal drugs [12]. In addition, potential benefits of combination therapy which include enhanced potency of antifungal efficacy, reduced selection of resistant organisms (particularly for flucytosine), and reduced toxicities due to lower dosing, are important to be taken into account.

Several chemical groups of plant constituents have been reported for their antifungal activity. Among them: some fatty acids, peptides and alkaloids, but it should be especially highlighted the groups of polyphenols and terpenoids which have furnished a huge variety of active structures. Interesting reviews have been published [9,12,38-40].

Examples of active compounds with different chemical structures, some of them the result of research conducted by our group, are discussed below.

3.1. Antifungal fatty acids

Some fatty acids have shown antifungal activity mainly due to their capacity to disrupt the bacterial membrane. They are able to interfere in the cell membrane structure displacing phospholipids and increasing its permeability. A well-known example is undecylenic acid, a semisynthetic product prepared from ricinoleic acid obtained from castor oil, the oil from seeds of *Ricinus communis*. Undecylenic acid is mainly used in the treatment of superficial mycoses.

During the search for new antifungals from Paraguaian plants, a series of fatty acids were isolated by our group from the bark of *Calycophyllum spruceanum* var. *multiflorum* (Griseb.) Chodat & Hassl. (Rubiaceae). Bio-guided fractionation of the dichloromethane extract afforded a mixture of eight fatty acids which were identified through GC-MS, HPLC-MS, ^{1}H- and ^{13}C-NMR analysis. Five of them were acetylenic: 6-hexadecinoic acid, 6-heptadecinoic acid, 6-octadecinoic acid, 6-nonadecinoic acid and 6-eicosinoic acid, whereas the other three were saturated: palmitic acid, heptadecanoic acid and stearic acid. Acetylenic fatty acids are very rarely

found in nature, and particularly three of them constituted new natural products: 6-heptadecinoic, 6-nonadecinoic and 6-eicosinoic acids. The mixture was active against the dermatophytes *Microsporum gypseum* and *Tricophyton mentagrophytes* with MIC and MFC values of 0.25 μg/mL, lower than those of the reference drugs nystatin (MIC and MFC values from 1.25 to 2.50 μg/mL) and amphotericin B (MIC and MFC values from 0.31 to 0.63 μg/mL) [41].

3.2. Polyphenols

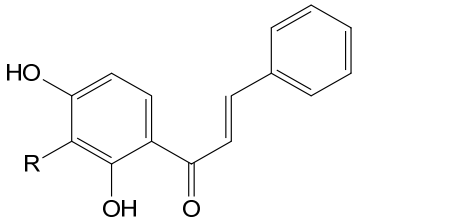
Many polyphenols, including simple phenols, flavonoids, coumarins, tannins and quinone derivatives, have been reported to have antifungal activity, which is partly dependent on the number and position of hydroxyl groups. They may act by inhibiting microorganism enzymes, possibly through reaction with sulfhydryl groups or through less specific interactions with proteins [12].

The antifungal spectra, potency and fungicidal properties of two chalcones from *Zuccagnia punctata* Cav. (Fabaceae), a plant used in traditional medicine in Argentina as antiseptic and wound-healing poultices, evidenced that they could have potential as antifungal agents for human beings. Particularly, 2',4'-dihydroxy-3'-methoxychalcone and 2',4'-dihydroxychalcone (Fig. 1) evidenced very strong activities against several clinical strains of the dermatophytes *T. mentagrophytes* and *T. rubrum* with MIC values in the range of 1.9-15.6 μg/mL and MFC values between 1.9 and 7.8 μg/mL. In addition, 2',4'-dihydroxychalcone showed a moderate activity against clinical isolates of the yeasts *Candida* sp. and *Cryptococcus neoformans*. Regarding their mode of action, both chalcones appeared to be fungicidal rather than fungistatic; moreover, 2',4'-dihydroxychalcone did not disrupt the fungal membranes up to 4×MFC and did not act by inhibiting the synthesis of polymers of the fungal cell wall. So, their mechanism would be different than that of most antifungal drugs in clinical use, i.e. amphotericin B, azoles or echinocandins [42].

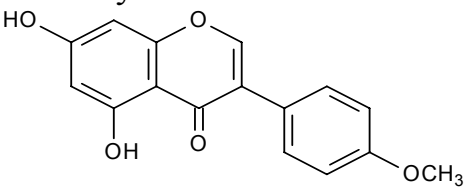
In the course of our search for new antifungals from Latin-American plants different active polyphenolic structures were identified in our laboratory, among which flavonoids, caffeic acid derivatives and lignans.

Two isoflavones, genistein and biochanin A, were isolated from the bark of *Andira surinamensis* (Boudt.) Splitz (Leguminosae) collected in Perú. It is a high tree mainly distributed in tropical America, whose bark is used in traditional medicine for different purposes. The dichloromethane extract, which showed a good activity against several fungal strains in the agar disk diffusion assay [15], was subjected to activity-guided fractionation yielding the two isoflavones. Particularly, biochanin A (Fig. 1) evidenced antifungal

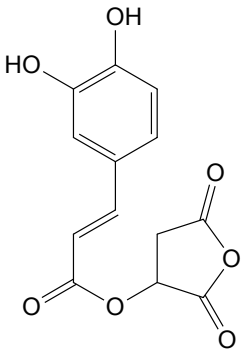
activity especially against yeasts and dermatophytes. Both isoflavones have previously been isolated from other *Andira* sp. [43] and are known to have antifungal activity [44]. In addition, the activity of the methanol extract against the dermatophyte *M. gypseum* and *T. mentagrophytes* was mainly related to the presence of condensed tannins [45].



R= -H 2',4'-dihydroxychalcone
R= -OCH_3 2',4'-dihydroxy-3'-methoxychalcone



Biochanin A



Malic anhydride caffeate

Figure 1. Some antifungal polyphenols isolated from Latin-American plants [41,42,45].

The isolation and identification of a new antifungal caffeic acid derivative was performed from the methanol extract of the aerial parts of *Geophila repens* (L.) I. M. Johnston from Paraguay, a Rubiaceae used by the Quechuas for the treatment of fungal infections [46]. In this case, the active methanol extract was subjected to partition with increasing polarity solvents, namely dichloromethane, ethyl acetate and butanol, the fraction obtained with the last solvent being the most active against *C. neoformans*, *M. gypseum* and *T. mentagrophytes*, its activity even persisting for one week in the agar disk diffusion assay. Fractionation by successive column chromatography on Sephadex® LH-20 led to the isolation of an ester of caffeic acid and malic anhydride identified as 2,5-dioxotetrahydrofuran-3-yl caffeate (malic anhydride caffeate) (Fig. 1), a compound not previously described, as the main responsible for the activity [47].

Piper fulvescens C. DC. is an herbaceous plant growing in Paraguay whose leaves are used in traditional medicine [48]. Activity-guided fractionation of their dichloromethane extract, which showed growth inhibitory activity against the yeasts *Candida albicans* and *Saccharomyces*

cerevisiae, as well as against the dermatophytes *M. gypseum* and *T. mentagrophytes* [15], led to the isolation of three neolignans identified as conocarpan, eupomatenoid 5 and eupomatenoid 6 (Fig. 2), for which antifungal activity had not previously been described. Conocarpan showed the broadest spectrum of activity, being active against yeasts and dermatophytes, whereas eupomatenoid 6 showed the strongest activity against *M. gypseum* and *T. menthagrophytes* with MIC values of 0.5 and 1.0 µg/mL, respectively. Moreover, eupomatenoid 5 turned out to be the least active. These results suggest that the absence of a methoxy group at 3' position of the phenyl-propenyl-benzofuran structure plays an important role in the antifungal activity, whereas the saturation of the C2-C3 bond influences the selectivity [49].

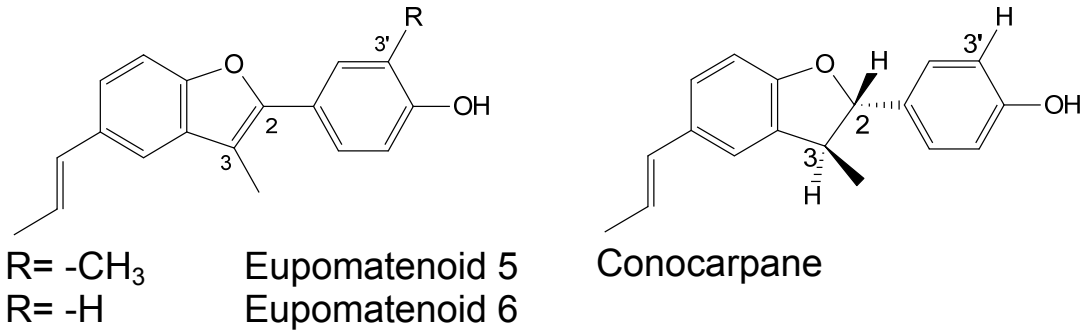


Figure 2. Antifungal neolignans from the leaves of *Piper fulvescens* [49].

3.3. Terpenoids

Among terpenes a variety of chemical structures showing interesting antifungal activities have been reported in recent years [9]. Their mechanism of action is not fully understood although it has been suggested to involve membrane disruption due to their lipophilic nature [12].

A high-throughput whole animal assay for the identification of antifungal compounds which uses the nematode *Caenorhabditis elegans* as a heterologous host [50] was performed to screen the activity of 2560 natural products against *C. albicans*. From all of them, those compounds that produced higher survival rates of the infected nematodes were polyglycosylated saponins with different types of skeletons. Two of them, a spirostanic one (aginoside) and a triterpenic one derived from barrigenol, were selected for further studies concerning their mechanisms of action. They inhibited *C. albicans* isolates at relatively low concentrations (16 and 32 µg/mL), including isolates resistant to clinically used antifungal agents, such as fluconazole and echinochandins. *C. albicans* hyphae and biofilm

formation were also disrupted by these two saponins at concentrations below the MIC. Furthermore, no hemolysis of erythrocytes was observed at three-fold the MIC for *C. albicans*, suggesting the saponins may have a preference for binding to fungal ergosterol when compared to cholesterol of the erythrocyte membranes [51].

One of the few antifungal products from plants that have been subjected to clinical trials is the methanol extract from leaves of *Solanum chrysothrichum* Schldh. (Solanaceae), a herbal drug used in Mexico for the treatment of fungi-associated dermal and mucosal infections, particularly recommended to cure *Tinea pedis*. *In vitro* assays showed antifungal activity against *T. mentagrophytes*, *T. rubrum* and *M. gypseum*, and a preliminar clinical study performed with the methanolic extract at 5% in a cream applied topically on patients affected of *Tinea pedis* showed clinical success with no side effects [52]. Later on, five new antimycotic spirostanic saponins were isolated and identified [53,54]. Clinical trials with a standardised methanol extract demonstrated not only the therapeutic efficacy and tolerability when applied topically as a cream against *T. pedis* [55] but also on *Pityriasis capitis* [56] and as suppository on *Candida* sp.-associated vaginal infection [57].

The screening on plant products with antifungal activity based on an ethnopharmacological approach, carried out by our research group, allowed the characterization of several antimycotic terpene compounds, for which antifungal activity had not previously been reported. Among them, the diterpene lactone acanthoaustralide and the sesquiterpene ester 6-cinnamoyloxy-1-hydroxyeudesm-4-en-3-one (Fig. 3) were respectively isolated through bio-guided fractionation from the dichloromethane extracts

Figure 3. The sesquiterpene ester 6-cinnamoyloxy-1-hydroxy-eudesm-4-en-3-one and the diterpene lactone acanthoaustralide are the antifungal principles of the roots of *Vernonanthura tweedieana* and the leaves of *Acanthospermum australe*, respectively [58,59].

from leaves of *Acanthospermum australe* (Loefl.) O. Kuntze and from roots of *Vernonanthura tweedieana* (Baker) H. Rob. (=*Vernonia tweedieana* Baker), two Asteraceae traditionally used in Paraguay for the treatment of skin diseases such as eczema and itching. Both compounds evidenced a strong activity against *T. mentagrophytes* with the same MIC and MFC values: 2 μg/mL in the case of acanthoaustralide and 4 μg/mL in the case of the sesquiterpene derivative [58,59].

3.4. Essential oils

Special consideration should be deserved to essential oils, many of which have been used for centuries for their antiseptic activity. Antimicrobial essential oils are nowadays used in the treatment of dermal and mucosal infections, such as acne, onychomycosis, vaginal infections, gingivitis or teeth and gums healthcare, as well as food preservatives and in the control of crop diseases.

A number of papers dealing with the antifungal activity of many essential oils have been published during the last decades, some of them discussing relationships between structures and activities and with interesting contributions on their mechanism of action, which is complex and has not yet been fully explained [39,40,60-66].

Different types of constituents of the essential oils have proven antimycotic properties (Fig. 4). Those that have reported the strongest activity are phenolic monoterpenes (e.g. thymol and carvacrol), phenylpropanoids (such as eugenol), alcoholic monocyclic monoterpenes (e.g. α-terpineol and terpinen-4-ol), and bicyclic monoterpene hydrocarbons (e.g. α-pinene) and ketones

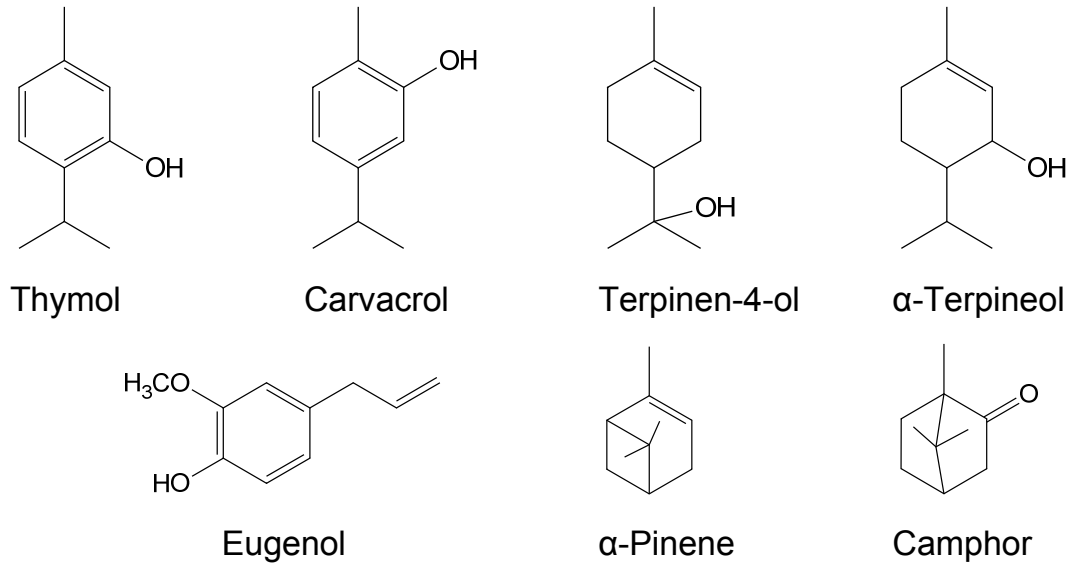


Figure 4. Typical antifungal compounds found in essential oils.

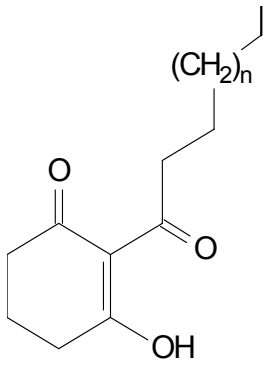
(e.g. camphor). The acidic nature of the hydroxyl group of phenols facilitates a hydrogen bond with enzyme active centres, being responsible of their high activity [67,68].

In the course of our research, several essential oils have demonstrated strong antifungal activities. The investigation of their composition together with bio-guided fractionation led to the identification of the main active constituents responsible for the activity.

From the antifungal essential oils from *Piper amalago* L. collected in Panama, a series of four new 2-acyl-3-hydroxycyclohex-2-en-1-ones, which represented more than 50% and 85% of the oils of the leaves and the stems, respectively, were isolated and their structures were established by EI-MS and NMR spectroscopy as 2-hexanoyl- (**1**), 2-octanoyl- (**2**), 2-decanoyl- (**3**) and 2-dodecanoyl-3-hydroxycyclohex-2-en-1-one (**4**) (Fig. 5). **1** and **2**, the two main constituents of the essential oils, showed a good antifungal activity against different yeasts. The hexanoyl- compound (**1**) showed the highest activity against *C. albicans* and *S. cerevisiae*, whereas the octanoyl-derivative (**2**) was the most active against *Candida lactis-condensi* and the decanoyl- one (**3**) only showed activity against *C. lactis-condensi*. These findings suggested that the length of the lateral chain modulates the activity [69,70].

The essential oil from fresh leaves of *Plinia cerrocampanensis* Barrie (Myrtaceae) from Panama, mainly constituted by oxygenated sesquiterpenes (65.9%), especially α-bisabolol (42.8%), bisabolol oxide B (10.3%) and *trans*-nerolidol (9.4%), was assayed against several fungal strains showing the strongest activity against *M. gypseum*, *T. mentagrophytes* and *T. rubrum* with MIC values from 32 to 125 μg/mL [71].

The unusual compound (-)-5,6-dehydrocamphor was found to be the major constituent in the essential oil from aerial parts of *Zuccagnia punctata* Cav. (Fabaceae) from Argentina, reaching percentages from 12.3% to 56.5%.



1: n=1 2-hexanoyl-3-hydroxycyclohex-2-en-1-one
2: n=3 2-octanoyl-3-hydroxycyclohex-2-en-1-one
3: n=5 2-decanoyl-3-hydroxycyclohex-2-en-1-one
4: n=7 2-dodecanoyl-3-hydroxycyclohex-2-en-1-one

Figure 5. Antifungal acyl-hydroxycyclohexenones of the essential oils from *Piper amalago* [69].

It was isolated from nature for the first time and its structure was completely elucidated from NMR data. The essential oil showed antifungal activity against the dermatophytes *M. gypseum*, *T. rubrum* and *T. mentagrophytes* with MIC values between 15.6-125 μg/mL, *T. rubrum* being the most susceptible species [72].

The composition and the antifungal activity of the essential oil from the rhizome and root of *Ferula hermonis* Boiss. (Apiaceae), a perennial shrub that grows on the Hermon mountain between Lebanon and Syria, evidenced this species as a potential source of interesting antifungal agents. The exhaustive analysis of the essential oil combined with successive bio-guided fractionations afforded different active products among which the unusual acetylenic compound 3,5-nonadiyne (Fig. 6), α-bisabolol and a fraction with 73% jaeschkeanadiol benzoate were the most active against the dermathophytes *M. gypseum* and *T. mentagrophytes*. Particularly, the fraction with the latter compound showed the strongest activity with MIC and MFC values superior or equivalent to those of the positive controls amphotericin B and nystatin [32].

The daucane aryl esters jaeschkeanadiol benzoate (teferidin) and jaeschkeanadiol *p*-hydroxybenzoate (ferutinin) (Fig. 6) were also isolated from hexane and dichloromethane extracts of the rhizome and root of *Ferula hermonis*. Determination of MIC and MFC values of both compounds evidenced a stronger antifungal activity for ferutinin than for teferidin. Particularly, *T. mentagrophytes* was the most sensitive strain with MIC and MFC values ranging from 8 to 256 μg/mL [73]. These results suggest that the presence of a phenolic hydroxyl in the acidic moiety of the ester, in the case

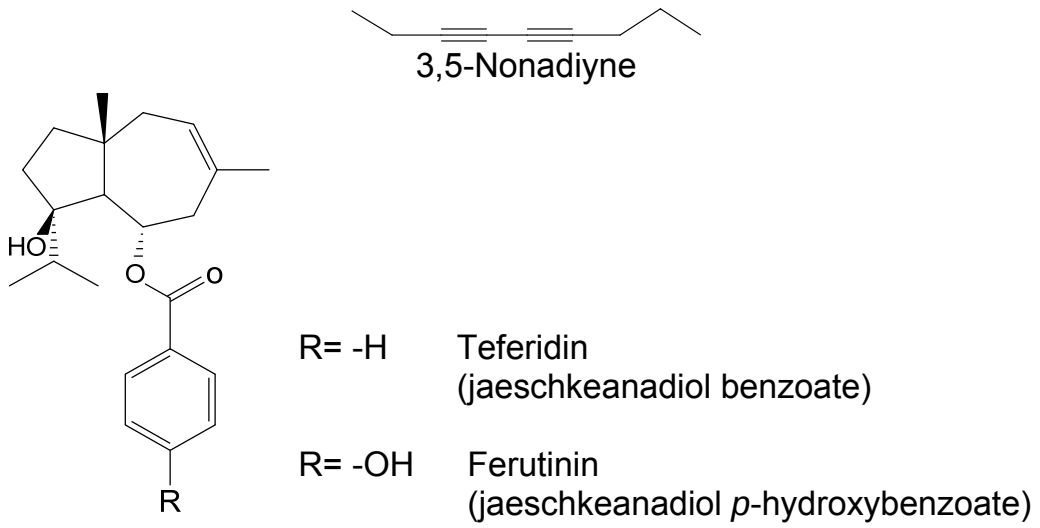


Figure 6. Antifungal constituents from the rhizome and root of *Ferula hermonis* [32,73].

of jaeschkeanadiol *p*-hydroxybenzoate, enhances the antifungal activity. This would be consistent with the fact that compounds with phenolic structures, like carvacrol, eugenol and thymol show stronger antimicrobial activities than those lacking a phenol group, as for example *p*-cymene [39,40,74].

Essential oils may induce visible signs of their action against fungi that can be observed as morphological changes both under microscope and as colony macro-features. Thus, eugenol and carvacrol have been found to cause significant deformations of the hyphae in food spoiling mould *Cladosporium herbarum*, which were related to their action on some cell wall enzymes, such as chitinases and glucanases [75]. Borneol induces morphological changes in cell walls and cellular organelles of *Aspergillus fumigatus* and *Epidermophyton floccosum* and inhibits mycelial growth of both filamentous fungi [76]. Furthermore, the essential oil from *Thymus vulgaris* inhibited mycelial development and germination of sporangiospores in *Rhizopus oryzae* [77], whereas the oil from *Zataria multiflora* caused vacuolisation of the cytoplasm and cell swelling, detachment of the cell membrane from the cell wall and deformation of mycelia in *Aspergillus flavus* [78]. Souza *et al.* [79] reported on the efficacy of *Origanum* essential oils against potentially pathogenic fungi, finding that the oil from *O. vulgare* completely inhibited radial mycelia growth of *T. rubrum* and *M. canis* and elicited disruption of cell structure in *Aspergillus flavus*.

Some studies evidenced that morphological modifications in the fungal cell may be induced even at sub-MIC concentrations [38]. In this way, Zuzarte *et al.* [62,65] found that essential oils from Portuguese *Lavandula* sp. completely inhibited filamentation in *Candida albicans* at concentrations as low as MIC/16. Also, farnesol derivatives prevent the conversion of yeast form into the invasive filamentous growth form of *C. albicans* at sub-inhibitory levels [80]. These findings could be of potential interest for the treatment of candidiasis.

Concerning their mechanism of action, as complex mixtures of a variety of chemical structures, essential oils do not seem to act on specific targets in fungal cells but to trigger different types of radical reactions [38,67,81].

Degradation of the cell wall and damage to both cytoplasmic and mitochondrial membranes have been proposed as the main mechanisms leading to changes in permeability and subsequent leakage and cell death [81]. It has been reported that lipophilic compounds accumulate in the lipid bilayers of the cell membrane altering its permeability and causing disruption of lipid packing and leakage of inorganic ions, while more hydrophilic constituents can enter the cytoplasm [38]. A mechanism of action related to damage of mitochondrial membranes and subsequent metabolic arrest rather than to direct disruption of cytoplasmic membrane or cell wall has been suggested by Zuzarte [65].

In the last years, investigations on the mechanisms of action of essential oils and their constituents showed that the antifungal activity is mediated through several mechanisms other than membrane disruption and inhibition of cell division. Inhibition of both ergosterol biosynthesis and H^+-ATPase leading to disruption of ion homeostasis and cell death [82-86] and induction of oxidative stress [87] have been proposed as main targets of antifungal essential oils. Several terpenoids inhibit microbial oxygen uptake and oxidative phosphorylation, the phenolic and non-phenolic alcohols exhibiting the strongest inhibitory effects, followed by aldehydes and ketones [66].

Synergistic effect between the essential oil constituents or between different essential oil mixtures against yeasts and dermatophytes have been reported by several authors [60,88,89]. But, due to increasing fungal resistance to classical antifungal drugs and their toxicity, what is more interesting from a clinical point of view is that combinations of essential oils or their pure constituents with commercial antifungal drugs used in therapeutics, such as amphotericin B or ketoconazole, can potentiate the activity of the latter and, thus, may lead to a dose reduction and minimization of secondary effects [90-94]. Although the combination of essential oils with other drugs can be very useful it has to be carefully considered, since depending on the percentage of each one antagonistic effects may also occur [40].

4. Conclusions

Despite the enormous progress made in recent decades in medicine, fungal infections are still an unsolved health problem, mainly due to the fact that the available antifungal drugs are of limited effectiveness. The plant kingdom is a source of medicinal preparations used in Traditional Medicine that offers a wide chemical diversity, making it of huge potential for new drug development. The research developed over the last years by different groups has led to the characterization of a variety of chemical structures with outstanding antifungal activity. Unsaturated fatty acids, polyphenols, triterpene derivatives and, particularly, essential oils have been shown as promising groups, some of them showing a synergistic effect with commercial antifungal drugs used in therapeutics. In case of complex mixtures such as essential oils synergy within some of their constituents have also been reported.

Acknowledgements

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3. *Cava* (Spanish sparkling wine) aroma: Composition and determination methods

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Abstract. *Cava* (Spanish sparkling wine) is one of the most important quality sparkling wines in Europe. It is produced by the traditional method in which a base wine is re-fermented and aged in the same bottle that reaches the consumer. The special ageing in contact with lees gives the *cava* a particular bouquet with toasty, sweet or lactic notes. These nuances could be related with the chemical composition of aroma. The methods required to analyze the flavor of *cava* are revised. Three approaches are necessary to obtain a wider profile: chemical, olfactometric and sensory.

Introduction

A sparkling wine is defined as one in which the carbon dioxide, produced exclusively by fermentation, is released when the bottle is opened. To obtain a European Union sparkling wine designation, the product must have excess pressure, due to carbon dioxide in solution, of no less than 3 bars when kept

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at a temperature of 20ºC in a closed container. In addition, the total alcoholic strength of the base wine (*cuvée*) used in sparkling wine preparation should not be less than 8.5% vol. For quality sparkling wines, the European regulation requires a minimum excess pressure of 3.5 bars and a minimum alcoholic degree of the base wine of 9% vol. [1]. The best quality sparkling wines produced in Europe are *Champagne* (from Reims, France), *Cava* (mainly from Penedès, Catalonia, Spain) and *Talento* (from Italy).

Cava is a Spanish sparkling wine produced by the traditional method (*Méthode Champenoise,* classical or traditional) in which a base wine is re-fermented in a sealed bottle [2]. The second fermentation is followed by an aging process in which the wine acquires its complex and particular bouquet. Aging is carried out in anaerobic conditions and always in contact with lees. Much of the chemical and sensory composition of *Cava* is acquired during aging. This evolution over time is attributed to yeast autolysis, the deterioration of yeast cells after their death, and the exchange between yeast cells and wine [3].

One of the first quality factors of a wine is its aroma. The analysis of aroma is a multidisciplinary science as it should involve three approaches: chemical composition, sensory analysis and an olfactometric study. All of these techniques are necessary to obtain an overall view of the aroma of wine. The characterization of aroma and its evolution during aging could be an interesting tool for winemakers. The present chapter describes the most useful methods for analyzing the aroma of sparkling wine and recent knowledge about the aroma of *cava* gained by our research group.

1. Méthode traditionelle or champenoise

C*ava* or Spanish sparkling wine is produced in two stages, following the traditional method. The first stage consists of white wine vinification and the second stage consists of a second fermentation in the same bottle that reaches the consumer, followed by a period of aging in contact with lees.

The first step begins with the harvest of the grape. The autochthonous V*itis vinifera* from Macabeu, Xarel·lo and Parellada varieties are the main grapes used in *cava* elaboration, which follows the typical vinification process of white wines. For high quality sparkling wines, must is obtained by soft pressure and is then clarified. The acidity of the must is corrected with tartaric acid or citric acid, and sulfur dioxide is also added.

Usually, alcoholic fermentation takes place in stainless steel tanks at a controlled temperature, which is always below 20ºC to prevent the loss of aroma molecules. The fermentation process takes a few days and results in a wine with no more than 1.5 g/L of residual sugar. Then, physical sedimentation

of colloids clarifies the wine; this can also be achieved by adding fining agents, such as bentonite.

If necessary, malolactic fermentation can be induced to decrease the acidity of the wine. Tartaric stabilization usually takes place by reducing the temperature of the tank below 0ºC in order to precipitate the tartrate crystals. The precipitate is usually eliminated by filtration. At this point in the process, the oenologist chooses the optimum blend or *coupage* of the monovarietal wines, in order to obtain the definitive base wine. The base wine composition is determined by the intended final product, the physicochemical composition (which must comply with the legislation) and the tasting notes, in particular.

The characteristic of the traditional method that distinguishes it from alternative methods (such as Charmat or Granvas) is the secondary fermentation in a closed bottle and the aging time in contact with yeast. A *liqueur de tirage* is added to the base wine, which is composed of yeast cells (yeast starter), sucrose, bentonite, and other compounds to stimulate yeast growth. The second fermentation of base wine is also called *prise de mousse*, because the carbon dioxide and effervescence are acquired in this phase. The second fermentation takes 2 or 3 months and can be considered finished when the sugar concentration is less than 1.5 g/L. Afterwards, the *cava* remains in contact with the lees for at least 9 months.

During ageing, autolysis of lees takes place. This involves the enzymatic self-degradation of cell components that is associated with cell death. The process leads to the release of some compounds to the wine and their interaction with wine constituents [4-6]. The wine is enriched by low molecular weight compounds and colloids [7], including nitrogen compounds, which are the most widely studied. In the first stages of autolysis, proteins and high molecular weight peptides are released to the wine and are subsequently hydrolyzed to smaller peptides and free amino acids [8]. The role of amino acids as aroma precursors is well known [9]. Degradation continues with the hydrolysis of glucans and mannoproteins [8]. Factors such as the time and temperature of *sur lie* ageing modulate the release of polysaccharides to the sparkling wine [7, 10]. In particular, the process of yeast autolysis is involved in the qualitative and quantitative composition of aromatic compounds [11].

The final stage of the traditional method begins with the riddling process, to eliminate the yeast lees from the bottle. It consists in bringing the yeast lees to the bottleneck through the addition of fining agents such as bentonite. The riddling process is usually performed with automatic riddlers, in which the bottles are placed in a cage and shaken to simulate the classical manual riddling process. Finally, less removal, which is called disgorging, is performed by submerging the neck of the bottle in a freezing bath of

polyethylene glycol (45%). The bottle is opened and frozen, and the lees come out of the top. Then a dose of expedition liqueur is added to restore the lost volume. The composition of the expedition liqueur defines the different commercial products on the market. Depending on the sugar added with the expedition liqueur, different indications must be cited on the label (Table 1). The last stage is closure with a cork, the capsule and the wire and the labeling of the bottle.

Table 1. List of indications for quality sparkling wine according to the sugar content.

Term	Conditions of use
Brut nature	Its sugar content is < 3 g/L; no sugar has been added after the secondary fermentation
Extra brut	Sugar content between 0 and 6 g/L
Brut	Sugar content < 12 g/L
Extra Dry	Sugar content between 12 and 17 g/L
Sec	Sugar content between 17 and 32 g/L
Semi-sec	Sugar content between 32 and 50 g/L
Doux	Sugar content > 50 g/L

During fermentation, and principally during ageing in contact with lees, the wine develops its characteristic foam and its particular bouquet. The factors that can influence the quality of sparkling wine and its chemical composition include the following: the grape varieties, the vineyard yield, the quality and composition of the base wine and the yeast strain. The second fermentation and the ageing in contact with yeast are key factors to explain the quality of sparkling wines like *cava* [8].

2. Aroma characteristics

Although the main attraction of sparkling wines is the CO_2, aroma is the sensorial aspect that is most appreciated by the consumer and is one of the main quality factors of a sparkling wine. The aroma substances that comprise flavors are found in nature as complex mixtures of volatile compounds. More than 6,000 compounds have been identified in the volatile fraction of foods, including over 800 in different types of wine. The concentration of these

volatile compounds varies from high concentrations (hundreds of mg/L) to lower levels (ng/L). The impact of each individual compound in the odor profile can be different. Most volatile compounds do not have a significant impact on flavor, although some that only occur in traces can have a significant influence. Here, the aroma properties of Spanish sparkling wine are reviewed.

According to some authors, such as Belitz *et al.* [12] or Ferreira *et al.* [13], volatiles from a wine can be classified according to the role they play, as follows:

- ✓ Genuine impact compound (individual compound associated with a specific aroma nuance)
- ✓ Major contributor (individual or family of aroma that provides a generic descriptor for the wine as citric or red wine. When they are removed, there is a qualitative change in the flavor of the wine)
- ✓ Net contributors (individual or family of aroma that provides a generic descriptor for a wine, but when they are removed only a decrease in the intensity of the descriptor is noticed)
- ✓ Secondary or subtle contributor (a compound found in wine at low concentrations, to provide an individually generic descriptor)
- ✓ Aroma enhancer (individual or family of aroma that does not provide the specific descriptor, but enhances the specific aroma of other molecules in the wine)
- ✓ Aroma depressor (molecule that decreases the intensity of an odor note)

In addition, wine volatile compounds can be classified in different ways: by chemical family (e.g. aldehydes, esters, ketones, terpenes, alcohols, norisoprenoids) or by sensory note (e.g. fruity, lactic, chemical, floral). However the most usual way to classify the aroma of wine is the stage in which the compounds are formed. The volatile compounds that can be found in sparkling wines come from grape (pre-fermentative origin), from yeast during the first or second fermentation or from ageing in contact with lees. This special ageing of *cava* provides a more complex profile, which is produced by chemical and enzymatic reactions.

The pre-fermentation volatile compounds depend largely on the grape variety. In addition, some compounds can be found that are formed by chemical or enzymatic reactions in must during grape processing (crushing and pressing). Other factors that have an influence are the degree of ripeness and the environment. The most usual varietal compounds are terpenes, which are typical of Muscat varieties, and methoxypyrazines, which are typical of *Cabernet sauvignon*, *Sauvignon blanc* and *Semillon* wines [14]. Methoxypyrazines provide

these varieties of wines with a characteristic note of green pepper. In the family of terpenes, the main compounds of interest in the olfactory field are alcohols and aldehydes. The monoterpenes can be found as free and glycosidically bound forms in grape berries [9]. The ratio between the free and bound form of monoterpenes varies during ripening, with a higher proportion of bound forms in mature grapes.

Carotenoids also play an important role in varietal aroma as precursors of C13-norisoprenoids such as TDN and vitispirane [15]. The oxidation of carotenoids releases some important strong odor fragments. According to some authors, these can be found in grape berries linked to a sugar molecule [16] or may be produced by direct carotenoid degradation [14].

Quantitatively, the most important portion of the aroma is that which comes from fermentation by yeast. Yeast influences the wine aroma as a consequence of microbiological transformations during alcoholic fermentation. One of the most important factors is the yeast strain of *Saccharomyces cerevisiae* that is used [17]. In addition, factors such as pH, temperature and must composition determine the biosynthesis of volatile compounds. Besides glycerol, ethanol, acetaldehyde and diacetyl, the main compounds formed are ethyl esters of fatty acids. Other fermentation compounds are higher alcohols that come from amino acid metabolism and associated ethyl esters, and volatile acids such as isovaleraldehyde, amyl alcohol, isobutanol, and 2-phenyl acetate. The Ehrlich reaction is responsible for the formation of these compounds.

Volatile esters are one of the most important classes of volatiles and are responsible for fruity notes in wine. The formation of these compounds differs according to the yeast strain [17], and factors such as fermentation temperature, nutrient availability and oxygen level must be taken into account [9]. In addition, the grape variety and the harvest year have a great influence on the volatile composition of wines. When six yeast strains were tested to evaluate the ability to produce optimum aromatic characteristics, those with high ethyl ester content showed a fresh sensory profile, with citric and green fruit notes, while the yeast strains that produce more acetate esters showed a profile with notes such as tropical and ripe fruit (data not shown).

Higher alcohols are related with yeast metabolism and, for this reason, wines fermented by different yeast strains can be differentiated [18]. Some authors, including Mateo *et al.* [19], have shown that the alcohol profile is dependent not only on the yeast strain but also directly on the inoculum concentration. According to these authors, the connection between the two chemical families, esters and higher alcohols, is related with the olfactory quality of wine:

$$[long\ chain\ esters] \times \frac{400}{[higher\ alcohols]}$$

This equation takes into account the relationship between esters and alcohols, according to Mateo *et al.* [19]. When the concentration of alcohol is above 400 mg/L is considered a negative quality factor.

The study by Torrens *et al.* [17] revealed that varietal compounds such as terpenes (linalool, citronellol), norisoprenoids (β-damascenone), and C_6 alcohols (hexanol and *cis*-3-hexenol) are linked with the yeast strain used in the vinification process. The liberation of terpenes and C_6 alcohols from their glycosylated conjugates is likely to be modulated by the enzymatic activity of the yeast strain.

However, it seems that other minority compounds, such as methionol or acetaldehyde, are independent of the yeast strain. Methionol seems to be produced by methionine catabolism and acetaldehyde is dependent on alcohol dehydrogenase activity and interaction with SO_2 concentration.

Finally, the third fraction of aroma is created during ageing in contact with lees. Usually, the evolution of aroma during ageing consists of loss of characteristic varietal notes and fermentation nuances, and the occurrence of the characteristic ageing notes, such as toasty and lactic [9, 18]. The process of autolysis seems to be a key factor in the development of the flavor profile of aged sparkling wines. Torrens *et al.* [18] compared the base wine and its corresponding young *cava* and reserve (> 15 months) to find the volatiles responsible for the characteristic and complex bouquet of *cava*. Three analytical methods were applied: physicochemical (SPME-GC-MS), olfactometric (GC-O) and sensory analysis, to obtain complementary information about the sensory profile of wines. When the sensory analysis was performed, the fruity profile of base wines evolved into a more complex bouquet with toasty, lactic and yeasty notes. These nuances were more pronounced in reserve than in young *cavas*.

When the behavior of volatiles in relation to ageing time was studied, some compounds were found to be useful as ageing markers. Acetate esters decrease during ageing [15, 20], while ethyl esters of high molecular weight, such as diethyl succinate and ethyl lactate, seem to be typical of aged *cavas* and could be used as age markers. Diethyl succinate, defined by the tasters as fruity or floral [18], could be a good ageing marker during the entire ageing period, while ethyl lactate was defined as a cheese note.

During ageing in contact with lees, the content of acetate esters decreases [15], which results in a loss of freshness of bouquet. According to some authors [21], ageing in contact with lees for 18 months has an impact on the

sensory profile, leading to enrichment with butter or vanilla aroma in white varieties. Torrens *et al.* [18] found that the lactic note increases significantly in reserve *cava*, which can be related with acetoin and diacetyl content.

In contrast, the content of some compounds of varietal origin, such as furans, thiol and norisoprenoids, increases during ageing [15, 18, 22]. Furans are derived from sugar degradation and they are described by tasters as toasted, dried fruit or caramel notes. Although they are not detected by GC, and only furfural has been identified and quantified, they play an important role in the bouquet of *cava*. Other extraction methods, such as SDE or CLSA, seem to be more adequate for the chemical determination of furan compounds [22]. The content of other varietal compounds such as lactones increases, according to Torrens *et al.* [18]. These compounds are difficult to identify and quantify in wines due to their low concentration, but they could be detected by olfactometric analysis due to their characteristic and desirable nuances (fruity, floral, and caramel).

In conclusion, ageing in contact with lees is a key factor in the development of the bouquet of *cava*. According to studies carried out to date, the bouquet of *cava* is described as toasty, lactic and sweet with enrichment in furan, lactone and norisoprenoid compounds in the volatile profile.

3. Aroma analysis

Aroma is one of the most important quality factors of a wine. Wine aroma analysis is a multidisciplinary science, which can be carried out following three principles: chemical analysis to obtain the qualitative and quantitative volatile profile, usually by means of gas chromatography-mass spectrometry; sensory analysis that uses people as the measuring instrument; and sniffing techniques that use gas chromatography coupled to an olfactometric detector.

3.1. Chemical analysis

The characterization of aromatic compounds is one of the toughest challenges in the analysis of *cava*. The main difficulty is due to the fact that the volatile fraction is composed of multiple substances of varying physical and physicochemical nature. However, the fact that the molecules are more or less volatile means that the instrumental technique of choice is gas chromatography (GC), preceded by an extraction phase and concentration of the volatile compounds. Since the 1950s, the most usual techniques for the determination of volatile composition are chromatographic analysis coupled with various detectors, such as the flame ionization detector (FID). Since the

emergence of mass spectrometry detection (MSD), which can be used to identify a large number of compounds, GC-MS has become the reference in aroma assessment. Before analysis, an extraction method is needed that is usually based on volatility (headspace techniques) or solubility (solvent extraction) of aromatic compounds, or a combination of the two.

Distillation methods including steam distillation (SD), simultaneous distillation-extraction (SDE), microwave-assisted extraction (MAE), and supercritical fluid extraction (SFE) have been previously applied to the analysis of wine aroma. In particular, SDE (the Likens-Nickerson technique) combines the advantage of liquid-liquid extraction with steam distillation techniques, and has been successfully used in wine analysis [22]. The use of solvents in the sample during the extraction increases the risk of contamination and the formation of artifacts. In addition, thermolabile compounds can be decomposed or transformed into other compounds. Prior to the chromatographic analysis, a concentration stage is needed to remove the solvent excess; this action can cause the loss of some volatile compounds.

Headspace techniques isolate volatile compounds according to their volatility. They mainly identify the most volatile compounds, so odor-active compounds are obtained. The method can be classified into two main kinds of extraction: static or dynamic headspace. Static headspace (SH) is based on static equilibrium under given conditions, and only a small fraction is extracted from the sample. The headspace composition depends on the partition of volatiles between air and the matrix in which they can be found [23]. SH usually gives a low yield of volatile compounds (the most volatile ones), so its combination with other techniques such as vacuum distillation is a good alternative. The use of dynamic headspace (DH) minimizes the low yield of some compounds [24]. One of the most commonly used DH techniques is the Purge-and-Trap analysis. The trapping stage of the analysis enhances the detection power of this technique. The stage consists of controlled flow of an inert gas, which sweeps the volatile compounds from the matrix. Then, they are trapped in an adsorbent where they will be retained until the following solvent extraction or thermal desorption [23].

In recent years, solid-phase microextraction (SPME) has become the most widely used method for food analysis, particularly for wine or sparkling wine [15, 22, 18, 25]. Initially, SPME was developed by Pawliszyn and coworkers in 1990 [26] for pesticide analysis in water. It is a solvent-free, simple, rapid, inexpensive and selective method for analyzing volatile and semi-volatile compounds. It can be applied in solid, liquid and gas matrices and is based on the equilibrium between the analyte concentration in the sample matrix and a stationary phase (a fused silica fiber coated with a suitable stationary phase) (Fig 1). Subsequently, target analytes are desorbed

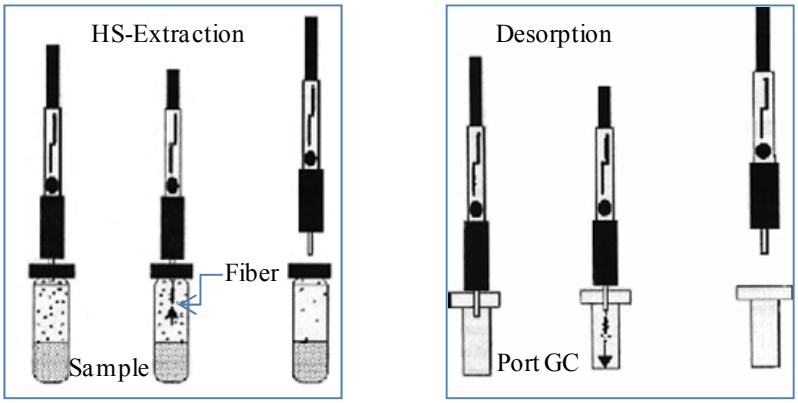

Figure 1. Scheme of the extraction and desorption process using the SPME technique.

immediately before chromatographic analysis by introducing the fiber into the liner of a GC-MS injector, normally in splitless mode (Fig. 1). The SPME can be applied by immersing the fiber into the sample (DI-SPME) or by exposing it to the sample headspace (HS-SPME).

The amount of analyte extracted by the fiber is determined by the partition coefficient between the sample matrix and the fiber coating. The fiber is coated by an absorbent polymer, such as Polydimethylsiloxane (PDMS) or Polyacrylate (PA), or an adsorbent polymer such as Carbowax (CAR) or Divinylbenzene (DVB). The fiber that currently provides the best results is the triple phase (CAR-DVB-PDMS), which combines three coatings with different polarities that can extract a wide range of compounds. Torrens *et al.* [25] found that this fiber had a better response; 76% more of the area than the other fibers tested (PDMS, PDMS-CAR and PDMS-DVB) in white and red wines aroma analysis.

3.2. Sensory analysis

Flavor perception is the result of multiple interactions between a wide range of chemical and sensory receptors located in the olfactory epithelium. The volatiles that reach the pituitary via the nose comprise the odor of a product, but when the chemicals reach the pituitary via retronasal stimulation, i.e. through the mouth, we talk about the flavor of the product [13]. Sensory analysis can be used to evoke, measure, analyze and interpret the reactions to stimuli perceived through the senses [27]. Sensory analysis still remains an efficient tool for assessing the properties of sparkling wine [21, 28, 29].

The current sensory analysis techniques can be divided into three categories: discrimination tests, consumer tests, and descriptive analysis. Discrimination tests are used to determine whether two products are perceived differently by human senses. The most common are the triangle test or the duo-trio, which is used when the differences between products are

very small. When the differences between products are greater and should be defined, the pair test is used to find out which sample is stronger in a specific attribute [30]. The triangle test is used in the oenology industry to evaluate the impact of the yeast strain used in the first fermentation on the sensory perception of *cava*. The triangle test is performed for the base wine, young *cava* and reserve *cava*. Fig. 2 shows that the influence of yeast strain on the differentiation of sparkling wine decreases with ageing time.

Other discrimination tests are the threshold test and the intensity ranking test. The threshold test is generally used to determine the importance of a component in a flavor, for example the taint of a product. The intensity ranking test is similar to a pair test, but more than two products are compared for a single attribute.

The second method that could be applied in the wine industry is the consumer test, also named the affective test, and is based on studying which product the consumer population likes. The affective test can be qualitative and quantitative. Usually, these tests are performed using a pair preference test between two samples. The scale used is hedonic or unstructured [27].

Finally, the most sophisticated tool in sensory analysis is descriptive analysis (DA). Descriptive analysis objectively characterizes and differentiates a product, using sensory descriptors. It has been used to analyze numerous alcoholic beverages, such as *champagne* [29], *cava* [18], gin [31], beer [32] and whisky [33, 34]. Descriptive analysis quantitatively characterizes appearance, aroma, taste and mouthfeel. The first requirement is to develop a lexicon to describe a target food. Vocabulary development is usually performed by a panel of experts from the industry, who have extensive experience in sensory analysis. The closed list used in the descriptive analysis is obtained by consensus in preliminary sessions and through discussion between the panelists and the panel leader. Synonyms, hedonistic or irrelevant terms must be eliminated from the preliminary list.

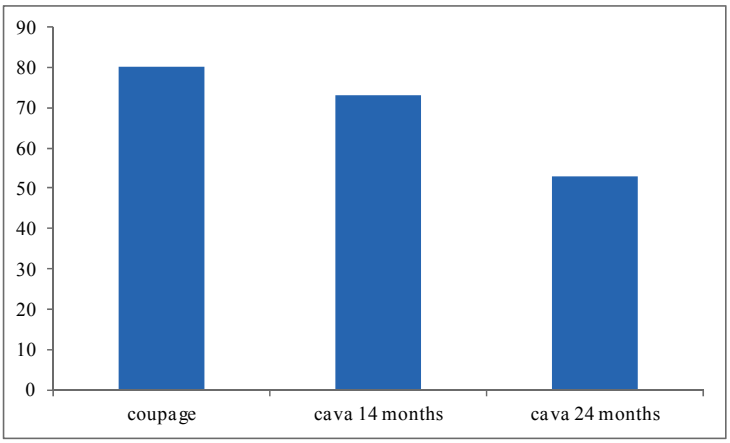

Figure 2. Percentage % of significant triangular tests among the six yeast strains [17].

According to some authors, the number of panelists required to perform a descriptive analysis is between 8 and 12. Panelists are trained with fortified wines at different concentrations [18] or through the qualitatively discrimination of artificial flavor standards [29]. The samples must be tasted at 20-22ºC to increase the perception of the aroma and flavor descriptors. In addition, quantitative descriptive analysis uses statistical tools, including multivariate methods such as principal components analysis (PCA) and ANOVA, to determine the appropriate terms, procedures and panelists for a specific product.

A few studies on sparkling wine (*cava* or *champagne*) can be found in the literature [18, 29]. The special ageing of sparkling wines through the traditional method in contact with lees leads to an evolution of the sensory profile different from that of table wines. The sensory profile of *cavas* was described empirically by the winemakers as toasted, yeasty and sweet.

Table 2 shows the olfactory descriptors evaluated by the panelists in the study by Vannier *et al.* [29] on *Champagne*, and by Torrens *et al.* [18] on base wine and *cava*. In the study by Vannier *et al.* [29], 16 descriptors were evaluated. In the study by Torrens *et al.* [18] on *cava*, fewer descriptors were used. In *cava* sparkling wines [18], the profile of the *cava* was more complex than that of the base wine, with toasty, lactic, yeasty and sweet nuances. These attributes are found to be significantly stronger in reserve *cava* than in young *cavas* (as confirmed by De la Presa-Owens, *et al.* [21]). In young *champagnes* the predominant attribute is herbaceous and in old *champagnes* the profile is similar to the profile of *cava*, with chemical and yeasty notes. In the same way, according to Vannier *et al.* [29], the exotic fruit notes decrease with ageing, while the butter and toasty notes increase in old *Champagnes*.

Table 2. Descriptors used in the studies by Vannier *et al.* [29] and Torrens *et al.* [18].

Champagne	Base wine	*Cava*	Reference aroma
Butter	Lactic	Lactic	Milk, cheese, butter
Caramel	Sweet	Sweet	Honey, caramel
Toast		Toast	Almond, nuts
Moss			
Floral	Floral	Floral	Rose, geranium,
Fruity lemon	Citrus fruit		Lemon, grapefruit
Fruit		Fruit	
Exotic fruits	Tropical fruit		Banana, pineapple
Ripe fruits	Ripe fruit		Jam, stewed fruit
Apple	Tree fruit		Apple, pear
Dust			
Herbaceous			
Animal			
Mould	Yeast	Yeast	Bread, baker's yeast
Spice			
Rubber	Chemical	Chemical	Petroleum, plastic

3.3. Olfactometric analysis (GC-O)

Olfactory GC techniques (GC-O) detect the influence of individual compounds on the sensory properties of food. Some compounds at very low concentrations (ng/L) can have a great impact on the aromatic profile, whereas other compounds at high concentrations may have very little influence.

GC-O is based on the sensory evaluation of eluate from the chromatographic column in order to detect the odor-active compounds. The judges who carry out olfactometric analyses are experts in wine aroma and are trained in the detection and approximate quantification of aromatic nuances. Usually, the same people perform the olfactometric and the sensory analyses. Olfactometric analyses can be used to detect whether a compound is odor active in the sample concentration, in which case the note is perceived by the judge, and the intensity of the odor [35]. The device consists of an olfactometric port connected in parallel with a conventional detector (Fig. 3). The eluate is split and reaches both detectors simultaneously in conventional detectors or with a slight time difference in mass spectrometer detectors. The available commercial ports are similar. An auxiliary gas (moist) is needed to prevent drying of the judges' nasal mucous membranes and the eluate passes the transfer line and is smelled in a conical port of PTFE fitted to the nose shape.

The appearance of olfactograms depends largely on the isolation method, as this affects the amount and composition of the final eluate. Isolation methods that reflect the release of volatile compounds from a food matrix can be considered more useful. In this context, static and dynamic headspace are useful, but the dynamic methods are used to a greater extent [36, 37, 38]. In the case of wine, the flavor profile is composed of a complex mixture of flavor compounds. To prevent the reduction of sensitivity due to fatigue, the analysis should be divided into several parts.

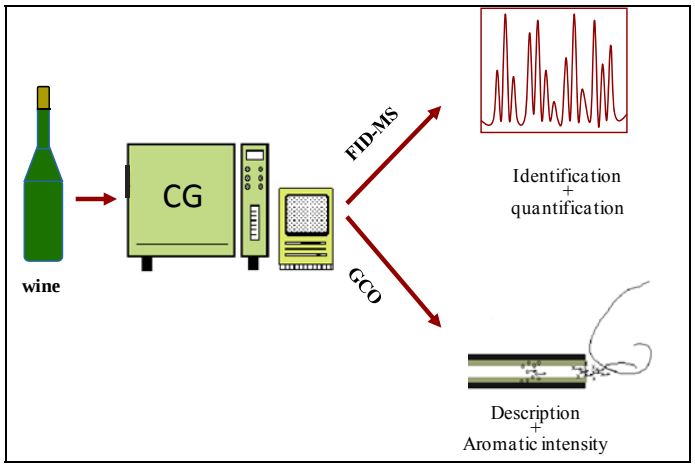

Figure 3. Schematic representation of GC-O analysis.

Various approaches are used to quantify volatiles in the sample:

1) Detection frequency methods (based on NIF or SNIF)
2) Dilution to threshold methods (CHARM and AEDA)
3) Direct intensity methods (including time-intensity, OSME and the finger span method)

In detection frequency methods (1), the percentage of judges who detect a determined odor at a determined retention time is calculated. The results are quantified using the nasal impact frequency (NIF) or surface of nasal impact frequency (SNIF), taking into account the stimulation time.

Dilution threshold methods (2) are the most commonly used techniques in analyses of alcoholic beverages. They include combined hedonic aroma response measurement (CHARM) and aroma extract dilution analysis (AEDA). These methods provide a quantitative description of the odor potential of a target compound, based on the ratio between its concentration in the sample and the sensory threshold in the air. The odor potential is usually counted as the odor activity value (OAV):

$$OAV = \frac{Concentration}{Sensory\ detection\ Threshold}$$

The AEDA method measures the highest sample dilution at which the odor is still detectable, while the CHARM method determines the duration of the odor sensation. Finally, direct intensity methods (3) measure the intensity and the duration of stimuli. The most usual is the odor magnitude specific estimation (OSME) method, in which the measure is dynamic: the appearance of an odor, the maximum intensity and the declination are all measured.

The methods that are usually employed in wine analysis [39] are the dilution to threshold methods CHARM and AEDA, and the direct intensity method OSME. However, only a few studies can be found about the olfactometric profile of sparkling wines such as *cava* or *champagne* [18, 36, 40].

4. Conclusion

Cava (Spanish sparkling wine) is a special wine produced mainly in the Penedès region (Catalonia, Spain) by the traditional method. Special ageing in contact with lees gives this beverage a particular bouquet with toasty, lactic and sweet notes. These nuances could be related with some chemical components, such as furan compounds with toast notes, lactones and norisoprenoids. The content of acetate esters decreases with ageing, which

reduces the more complex fruity notes in the base wines (tropical, tree and ripe fruits) so that the flavor evolves to a single note in the *cava*.

Due to the complexity of aroma, three approaches are required to determine the bouquet of *cava*: chemical, sensory and olfactometric. Further studies are needed to deepen knowledge of the compounds responsible for the flavor and notes of a product as complex as *cava*.

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4. Membrane interaction of polymyxin B and synthetic analogues studied in biomimetic systems: Implications for antibacterial action

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Abstract. Membrane-active antimicrobial peptides, such as polymyxin B (PxB), are currently in the spotlight as potential candidates to overcome bacterial resistance. We have designed synthetic analogs of PxB in order to determine the structural requirements for membrane action. Since the mechanism of action of PxB involves interaction with both the outer membrane and the cytoplasmic membrane of Gram negative bacteria, we have used an approach based on mimicking the outer layers of these membranes using monolayers, Langmuir-Blodgett films and unilamelar vesicles, and applying a battery of biophysical methods in order to dissect the different events of membrane interaction. Collectively, results indicate that the PxB analogues act in the bacterial membrane by the same mechanism than PxB, and that cationic amphipathicity determines peptide activity.

Introduction

The global emergence and spread of multidrug-resistant bacteria (defined as resistant to three or more antibacterial drug classes) is an important clinical

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problem. Some factors that have contributed to the development of bacterial resistance include inappropriate use of antibiotics, such as the overuse of powerful, broad-spectrum antibiotics, the presence of antibiotics in the food/livestock industry and the inclusion of antimicrobials in household products [1, 2]. The World Health Organization recognizes antimicrobial resistance as one of the three greatest threats to human health [3]. However, the antimicrobial pipeline remains unacceptably lean, in fact, the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species) have outpaced the drug discovery process [4, 5]. The number of antimicrobial agents approved by the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA) has sharply decreased over the last 25 years (Fig. 1). This has left few treatment options for the most stubborn forms of MRSA (methicillin-resistant *Staphylococcus aureus*). The picture is even bleaker for infections caused by Gram-negative bacteria, which are occasionally resistant to all the antibiotics now in the market. All these facts have made it necessary to launch different initiatives and efforts towards discovering new, safe and effective antimicrobials in the next decade [6]. All this work is beginning to crystallize, and in the last decade 20 new antibiotics have been launched and approximately 40 compounds are currently in active clinical development [7].

In this context, there is now renewed interest in the search for drugs that have more than one target on the bacterial cell, rather than a specific chiral receptor or enzyme, with a focus on modes of therapy with mechanisms less likely to promote resistance. Antimicrobial peptides (AMPs) are a class of antibiotics that has attracted great interest in the last few years because they rarely spur the development of resistant organisms. They are a diverse group of molecules that share a few common features, the most important is that their main target is the lipid bilayer itself, and in consequence to develop genetic resistance is very costly for bacteria.

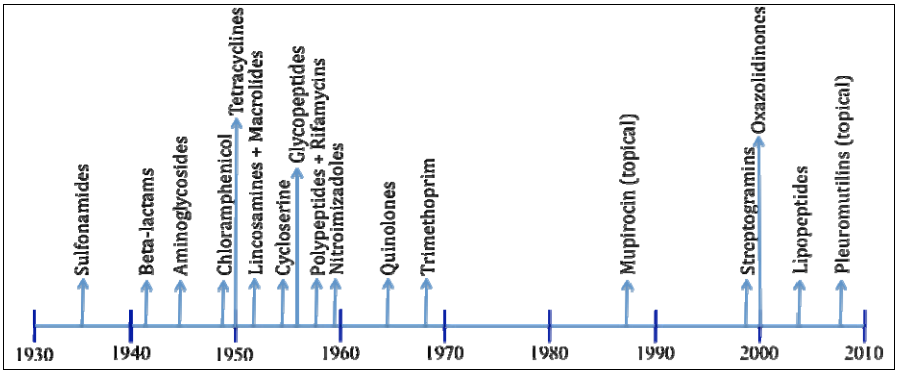

Figure 1. Between 1935 and 1968, 14 classes of antibiotics (with novel mechanisms of action) were introduced for human use; since then only 5 new classes have been described.

1. Antimicrobial peptides (AMPs)

Antimicrobial peptides are natural weapons produced by a variety of organisms, from single-celled microbes to vertebrates [8]. They are very diverse in their sequences, length (generally short) and structures (some of them are linear while others are cyclic due to disulfide bridges, with a variety of secondary structures) but they all adopt an amphipathic conformation with opposing hydrophobic and polar charged faces that allows them to interact and disrupt selectively negatively charged microbial membranes. They can be classified as follows [9]:

- Anionic peptides, such as dermcidin from humans and daptomycin.
- Linear cationic α-helical peptides, as for example magainin 2 and cecropins.
- Anionic and cationic peptides enriched for specific amino acids, like tryptophan-rich indolicin from cattle and Dab-rich polymyxins.
- Peptides that contain cysteine and form disulphide bonds, such as defensins and protegrins.
- Peptide fragments of larger proteins, as for example lactoferricin from lactoferrin.

Multiple studies have shown that AMPs have very good activities (in the micromolar range), and broad spectrum of activity against a range of bacteria, fungi, enveloped viruses and parasites [10] and represent a promising novel class of antibiotics. Traditionally, the search of novel AMPs involved the identification of active peptides from natural sources followed by the design of synthetic peptide analogs, but nowadays design is accompanied by structure-function studies, or *de novo* design that includes high-throughput combinatorial library screening, structure-based modeling, predictive algorithms and the introduction of non-coded modifications to conventional peptide chemistry [11]. Some AMPs have even been discovered through sequence searches within large proteins (such as lactoferricin or hemoglobin). Some examples of AMPs introduced or in advanced stages of development can be found in Table 1.

2. Mechanism of action of AMPs

Although the mode of action of antimicrobial peptides involves disrupting the integrity of the bacterial membrane in different ways, new evidence points to intracellular targets, including DNA and protein synthesis, protein folding, enzymatic activity and cell wall synthesis. In any

Table 1. Representative antimicrobial peptides in different stages of the drug-discovery process (M = Marketed).

Name	Description	Activity	Phase	Observations
Polymyxin B (PxB)	10-amino acid lipopeptide	Antibacterial Gram-	M	First discovered in the 1940s, but their use was discontinued due to nephrotoxicity and neurotoxicity
Colistin (Polymyxin E)	10-amino acid lipopeptide	Antibacterial Gram-	M	Discovered in the 1950s, differs in 1 amino acid from PxB, less active than PxB but also less toxic and so preferentially used.
Nisin	34-amino acid polycyclic peptide	Antibacterial Gram+	M	In 2001, approved as antimicrobial food additive
Daptomycin	13-amino acid lipopeptide	Antibacterial Gram+	M	Originally developed by Lilly in the early 1980s but abandoned after early clinical trials. Cubist Pharmaceuticals launched daptomycin in 2003
Telavancin	lipoglycopeptide	Antibacterial Gram+	M	Approved by the FDA in 2009 for complicated skin and skin structure infections
Pexiganan (MSI-78)	22-amino acid analogue of magainin 2	Broad-spectrum antibacterial	III	No advantage was demonstrated over existing therapies
Iseganan (IB-367)	synthetic protegrin 1	Broad-spectrum antibacterial and antifungal	III	No advantage was demonstrated over existing therapies
Omiganan (MBI-226)	synthetic analogue of indolicidin	Broad-spectrum antibacterial and antifungal	III	Failed in phase III of catheter-related infections but is actually under Phase III trials for acne and rosacea
PAC-113	12-amino acid peptide based on histatin	Candidiasis (*C. albicans*)	II	Phase I and Phase II completed with promising results, no Phase III trials proposed yet
XOMA 629	9-amino acid peptide derived from bactericidal/permeability-increasing protein (BPI)	Impetigo	II	First tested for acne without demonstrating benefit over existing therapy, actually in Phase II trials for treatment of impetigo
HB1345	synthetic lipohexapeptide	Acne (*Propionibacterium acnes*)	Pre-clinical	Promising due to short length

case, membrane interactions are important even for intracellular-targeting peptides, because they must translocate the lipid membrane. Diverse studies have indicated that the interaction of AMPs with the bacterial membranes is specifically based on their structural properties. It is thus their sequence, size, cationic nature, hydrophobicity and amphipathicity that govern their interaction with target cells [12, 13].

According to the Shai-Matsuzaki-Huang (SMH) model [14-16], unstructured peptides adopt a three-dimensional structure when interacting with the bacterial membrane and fold into amphiphilic molecules, with positively charged sides directly interacting with the anionic lipid head-groups of the bacterial membrane. The lipids are then displaced by the peptides, causing a thinning of the outer leaflet of the bacterial membrane. Right after this, the peptides aggregate and form a channel that allows for peptide translocation into the interior of the target cell (Fig. 2). At this point, there are three different models that have been proposed to explain the method of pore formation; the barrel-stove model, the torodial pore (wormhole) model and the micellisation model [17, 18]. There does not appear to be one model that fits all peptides, with various factors contributing to the process of pore formation. The actual antimicrobial activity in order to

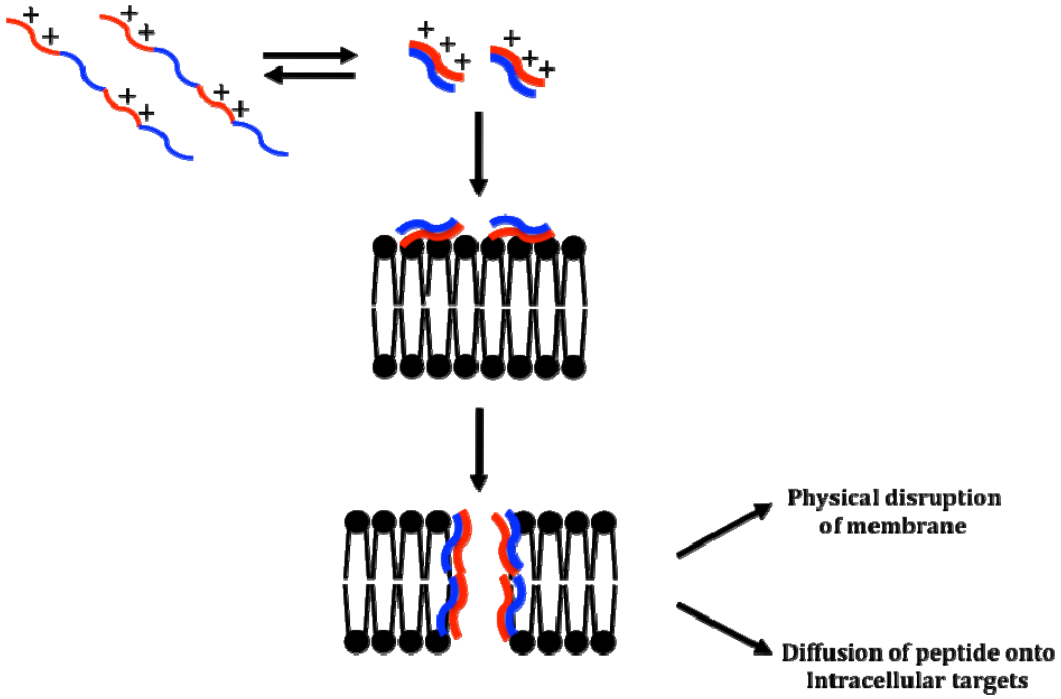

Figure 2. The SMH model for the mechanism of action of an AMP. The various steps include: carpeting of the outer leaflet, insertion of the peptide into the bilayer and finally collapse of the membrane or in some cases diffusion of the peptide to target the intracellular target.

kill bacteria resides in part in the disruption of the bacterial cell membrane, which can occur in a number of ways. These include membrane depolarization, permeabilization or creation of pores which could cause cellular contents to leak out, degradation of cell walls, and alteration of the lipid composition in the membrane bilayer which could lead to the disturbance of membrane functions [8, 19].

A variety of techniques have been used to assess the mechanisms of antimicrobial peptide activity. Each method provides a slightly different view of peptide activity and no single technique is capable of adequately determining the mechanism of action of AMPs. Such techniques include:

- Biophysical studies with model membranes: To provide insights into mechanisms of activity and therefore determine the type of damage induced by the peptides, we can study the interaction of AMPs with phospholipids in model membranes (liposomes or monolayers), of single or mixed lipids in order to mimic the different composition of bacterial membranes. The use of fluorescent dyes can also be useful, such as encapsulated dyes to detect the formation of pores or labeled lipids to determine the effect on the bilayer [13].
- Microscopy: The use of microscopy techniques to visualize the effects of AMPs on microbial cells has helped identify general target sites. Scanning and transmission electron microscopy have been used to demonstrate the ultrastructural damaging effects of antimicrobial peptides [20]. This analysis has shown that different peptides have different effects on microbial cells, indicating that they might have different target sites or mechanisms of activity.
- Circular dichroism (CD) or oriented circular dichroism (OCD): The orientation and secondary structure of an AMP bound to a lipid bilayer can be measured by CD in a controlled humidity environment with light incident normal to the sample surface [21].
- Flow cytometry (FC): Flow cytometry in conjunction with different dyes can be used to determine the effects of the peptides on bacteria, such as propidium iodide (PI) to detect membrane permeability or bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] to detect depolarization of the membrane [22].
- Solid-state NMR spectroscopy (ssNMR): Solid-state NMR measures the secondary structure, orientation and penetration of AMPs into lipid bilayers in the biologically relevant liquid-crystalline state [23]. These data help define the interactions of AMPs with bacterial membranes and the effects of peptide and membrane composition on activity.

3. Mechanism of action of polymyxins

Emergence of nosocomial bacterial pathogens with acquired resistance to almost all available antibiotics, namely "superbugs", is a growing medical concern. The major challenge is regarding the treatment of multidrug-resistant Gram-negative bacteria, such as *Pseudomonas aeruginosa* or *Acinetobacter baumannii*, since no new antibiotic is even in the drug development pipeline with the sole exception of tigecycline. Consequently, there has been a resurgence of old antibiotics, such as polymyxins discovered in the 1940s [24], as drugs of last resort for the treatment of infections caused by MDR Gram-negative pathogens, despite their toxicity (nephro- and neurotoxicity) and the lack of clinical efficacy data [25, 26].

Polymyxin B (PxB) and polymyxin E (colistin) are two secondary metabolite nonribosomal cyclic lipopeptides produced by the soil bacterium *Bacillus polymyxa,* and are the two polymyxins used clinically. Both are specific and highly potent against Gram negative bacteria. They share a common primary sequence, with five positives charges due to L-α,γ-diaminobutyric acid (Dab), the only difference being at position 6, which is occupied by D-Phe in PxB and D-Leu in colistin (Fig. 3).

The mechanism of action of polymyxins, and in particular of PxB, has been studied in detail [27]. The initial target is anionic lipopolysaccharide (LPS) in the outer membrane (OM) of Gram negative bacteria, resulting in OM permeabilizing action. The high affinity for LPS binding can be attributed to the polycationic character of PxB together with its amphipathicity, and the polar and hydrophobic domains folding in two distinct faces when binding to the bacterial membrane. LPS binding results in

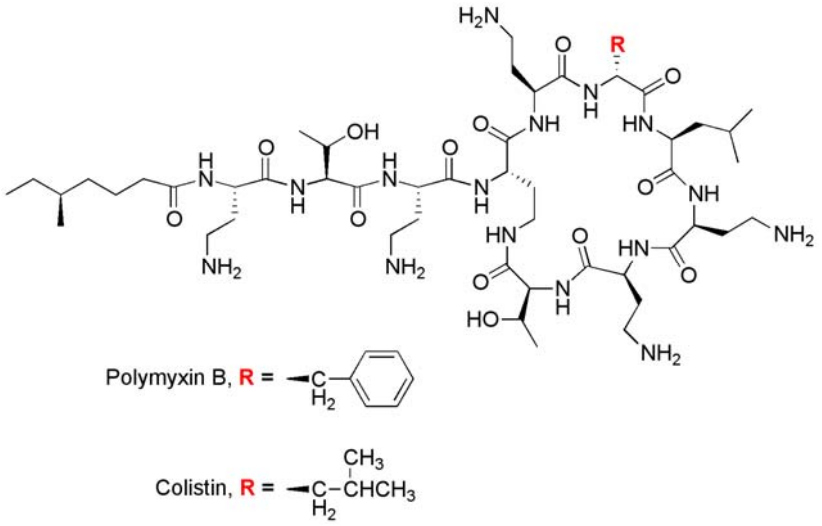

Figure 3. Structures of polymyxin B and colistin.

divalent cation displacement, and leads to PxB self-promoted uptake into the periplasmic space [28]. This process is necessary but not sufficient for bacterial killing, and subsequent interaction with the cytoplasmic membrane is necessary for antibiotic activity [28, 29]. The next step remains unclear at the molecular level, but biophysical studies with model membranes have given insight into the mechanism of bacterial killing by PxB. One possibility involves the insertion of PxB into the cytoplasmic membrane and the disruption of its integrity via membrane thinning or pore formation. However, several reports clearly show that permeabilization or depolarization of the membrane take place at concentrations of polymyxin well above the minimal inhibitory concentration (MIC), and thus other mechanisms of bactericidal action should be considered [12].

A more consistent mechanism of action is based on the formation of periplasmic membrane contacts between outer and inner membranes. According to this model, once in the periplasmic space PxB forms contacts between the two enclosed phospholipid interfaces and promotes a fast exchange of certain phospholipids. The resulting changes in the membrane lipid composition trigger an osmotic imbalance that leads to bacterial stasis and cell death. This phenomena has been proposed as the mechanism of antibacterial action of PxB and several other antibiotic peptides, including cecropins [30-32], and has major implications in bacterial resistance, since it would not be susceptible to generate stable genetic resistance. In fact, acquisition of resistance by a sensitive microbial strain against antimicrobial peptides is surprisingly improbable [8].

Biophysical studies using model membranes have demonstrated that at the concentrations around the MIC, PxB induces the apposition of anionic vesicles and the formation of functional vesicle-vesicle contacts that support a fast and selective exchange of phospholipids exclusively between the outer monolayers of the vesicles in contact and maintaining intact the inner monolayers and the aqueous contents, as schematized in Fig. 4 [33-35].

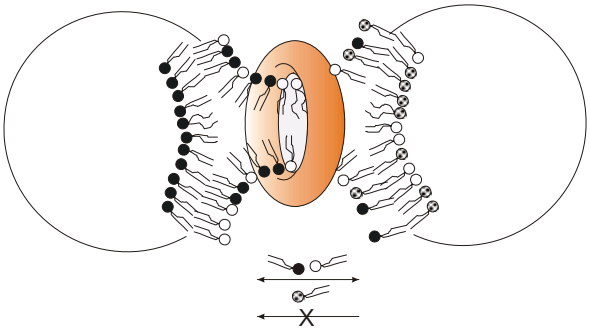

Figure 4. Model showing the formation of membrane contacts mediated by PxB. Key points are: *(i)* contacts involve only the outer monolayers of the apposed vesicles; *(ii)* bilayer structure is maintained; *(iii)* lipid transfer is selective and depends on the lipid headgroup composition; *(iv)* PxB is accessible from the aqueous phase; *(v)* contacts are stable (from [33-35]).

A battery of physicochemical and biophysical methods has been applied to dissect and study the different events of the interaction of polymyxin with the lipid membrane. Overall, these methods amplificate the effect caused by very few molecules of peptide bound to the membrane. Unilamelar vesicles and monolayers at the air/water interface have been prepared with lipopolysaccharide (LPS from *Salmonella minnesota*) to reproduce the outer membrane of Gram-negative bacteria. To mimic the cytoplasmic membrane, mixtures of 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphoglycerol (POPG) and 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphoethanolamine (POPE), or lipid extracts of *Escherichia coli,* have been used.

Lipid monolayers are valuable simplified models of biomembranes, and have been extensively used in the study of the interfacial properties of membrane active peptides. Monolayers are bidimensional model membranes with a planar geometry where the lipid molecules have a defined orientation, and are very suitable for thermodynamic analysis [36]. Transferred to a solid support, lipid or lipid-peptide monolayers can be investigated by atomic force microscopy (AFM), a powerful technique to study the structure and properties of the lipid surface at a nanometric scale [37].

Thermodynamic analysis of mixed monolayers formed by PxB and *E. coli* lipids shows non-ideal mixing consistent with lipid-PxB domain formation [38]. At very low mole fractions of PxB at the interface, below 0.04 mol fraction, monolayers had associated small positive values of excess Gibbs energy, thus suggesting immiscibility and formation of PxB clusters in the dominant lipid phase. AFM observation of transferred mixed and pure monolayers at the membrane equivalence pressure reveals that at the low concentrations of PxB required for contact formation, $X_{PxB}<0.04$, the antibiotic interacts with the bacterial lipids in a very unique way, inducing the formation of well-defined microdomains that are clearly distinguishable from the structures formed at lytic concentrations of PxB (Fig. 5).

Interaction with LPS monolayers was also determined by the Langmuir trough technique, maintaining constant either the surface pressure or the area. In addition, morphological changes in the monolayer upon peptide insertion have been observed by Brewster angle microscopy (BAM). BAM images of monolayers of PxB-containing LPS or its anchoring part, lipid A, show that PxB induces important changes in the film morphology with the formation of more condensed phases at lower surface pressures [39].

4. Polymyxin B synthetic analogues

In view of the excellent antimicrobial activity of the naturally occurring polymyxins and relatively low prevalence of resistance, it is not surprising that there has been substantial effort into discovery of polymyxin analogues with improved microbiological, pharmacological, and toxicological profiles [27].

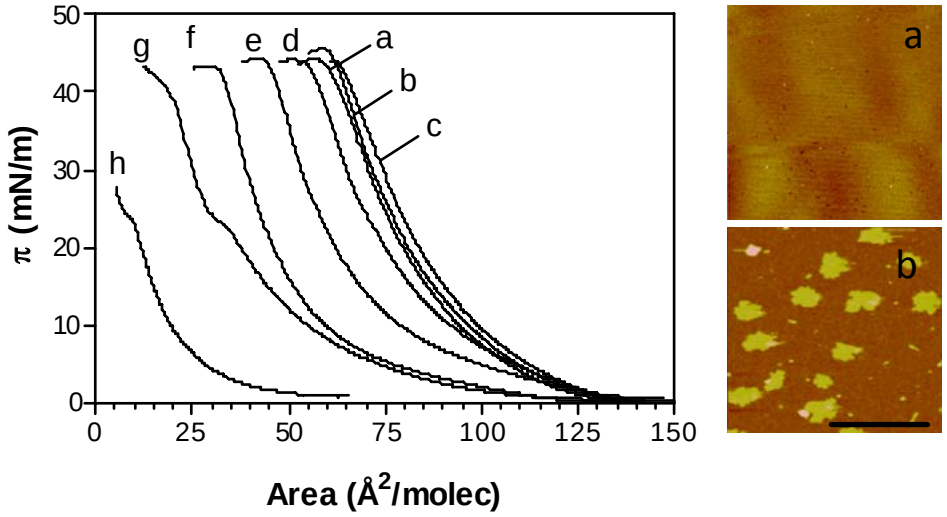

Figure 5. Variation of the surface pressure with the mean molecular areas of mixed ECL-PxB monolayers at various peptide mol fractions: (a) 0; (b) 0.005; (c) 0.01; (d) 0.17; (e) 0.36; (f) 0.56; (g) 0.77; and (h) 1. AFM images of the ECL-PxB L-B monolayers at 32 mN/m are shown at the right side. Bar represents 500 nm [38].

We have designed and synthesized a series of PxB-analogues that mimic the primary and secondary structure of PxB [39-43]. Peptide synthesis was performed manually following standard Fmoc/^{t}Bu procedures, using DIPCDI/HOBt activation on a benzhydrylamine resin. Homogeneity of peptide crudes was assessed by analytical HPLC, and peptides were subsequently purified by preparative HPLC. Cyclization of peptides through disulphide bonds was carried out by air oxidation. Final purity was greater than 95%. Peptides were characterized by amino acid analysis with a Beckman 6300 analyser and by MALDI-TOF with a Bruker Model Biflex III.

The first analogue obtained by this method (sp-B, structure in Fig. 6), closely mimics the primary and secondary structure of PxB. However, to ensure the economic viability of the potential new drug, the simplicity of the synthetic process had to be considered. For this purpose, the complex bond between Dab^{4} and Thr^{10} of natural PxB was substituted by cyclization via an intramolecular disulphide bridge resulting from oxidation of two cysteine residues inserted in the primary amino acid sequence, as had previously been reported [44], and linear nonanoic acid was introduced at the N-terminus.

Penetration of sp-B into monolayers of LPS gives information on the affinity of the lipopeptide for the main component of the Gram negative outer membrane [39]. Experiments conducted at constant pressure or at constant area showed that sp-B readily inserted into LPS monolayers, with even higher levels of affinity than the parent compound.

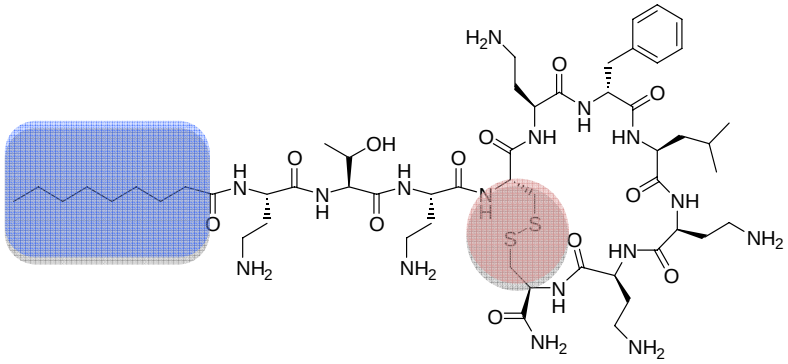

Figure 6. Sequence of PxB synthetic analogue sp-B. The two principal changes introduced to simplify the synthesis are highlighted.

Biophysical studies with unilamelar vesicles that model the cytoplasmic membrane reveal that low concentrations of sp-B form functional vesicle-vesicle contacts that are selective for transfer of anionic phospholipids [40, 41]. For example, fluorescence experiments based on the excimer/monomer ratio of pyrene-labeled phospholipids show that only monoanionic phospholipids such as 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphoglycerol (POPG) are transferred, whereas dianionic phospholipids such as 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphate (POPA) are not.

This interaction is equivalent to the previously described for PxB on anionic membranes [35], and therefore sp-B is a valid scaffold to introduce different structural changes with the objective of getting more insight into the molecular basis of the interaction of the peptides with the lipid membrane. In this sense, analogues sp-C and sp-D (sequences in Table 2) were designed and synthesized. The contribution of the hydrophobic domain (D)Phe^{6}-Leu^{7}, highly conserved in the polymyxin family, was assessed with analogue sp-C, where a permutation in the sequence disrupted this hydrophobic domain. The influence of the peptide charges on the interaction has been explored with sp-D, an analogue that contains two residues of L-α,γ-diaminopropionic acid (Dap) in positions 1 and 8. Dap residues have a pK_a approximately of 8.5 and at pH 8.0 will confer the peptide a much lower cationic character compared to PxB (the pK_a of Dab is 9.45).

Table 2. Peptide sequences.

Peptide	Sequence
PxB	R_1-Dab-Thr-Dab-*cyclo*[Dab-Dab-*(D)*Phe-Leu-Dab-Dab-Thr]
PxB-np	Thr-Dab-*cyclo*[Dab-Dab-*(D)*Phe-Leu-Dab-Dab-Thr]
sp-B	R_2-Dab-Thr-Dab-*cyclo*-[Cys-Dab-(*D*)Phe-Leu-Dab-Dab-Cys]
sp-C	R_2-Dab-Thr-Dab-*cyclo*-[Cys-Dab-(*D*)Phe-Dab-Leu-Dab-Cys]
sp-D	R_2-Dap-Thr-Dab-*cyclo*-[Cys-Dab-(*D*)Phe-Leu-Dap-Dab-Cys]

[a]R_1, (*S*)-6-methyloctanoyl ; [b]R_2, nonanoyl; sp-B, sp-C, and sp-D are cyclized by means of a disulfide bond between Cys-4 and Cys-10.

Results in this work are summarized in Fig. 7, and show that insertion into the LPS layer is achieved to different extents by all the lipopeptides assayed, including acyl chain-lacking compound PxB-np, which corresponds to a deacylated PxB decapeptide (Table 2). However, insertion into the anionic phospholipid membrane and adoption of an active form of the peptides, i.e. the ability to induce phospholipid exchange, is more restrictive, and requires the interplay of the two hydrophobic domains of the lipopeptide (the acyl chain and the D-Phe-Leu region), as well as the five positive charges of Dab.

In another study, synthetic fluorescent analogs of sp-B were used to investigate the peptide position and orientation in the intermembrane contacts: sP-Bw, an analog that contains *D*-tryptophan (*D*Trp) instead of the naturally occurring *D*-phenylalanine, and sP-Bpy, incorporating a pyrene group at the N-terminus [41]. The inclusion of *D*Trp is very useful to characterize binding of the peptide to the membrane by fluorescence techniques. Tryptophan fluorescence, anisotropy and quenching measurements performed with sP-Bw indicate that the peptide binds and inserts in anionic vesicles of POPG and POPA adopting different forms. sP-Bw also serves as a donor for intermolecular resonance energy transfer (FRET) experiments with sP-Bpy used as acceptor. FRET experiments are consistent with self-association of sP-B bound to anionic vesicles at the concentrations that induce the vesicle-vesicle contacts (Fig. 8).

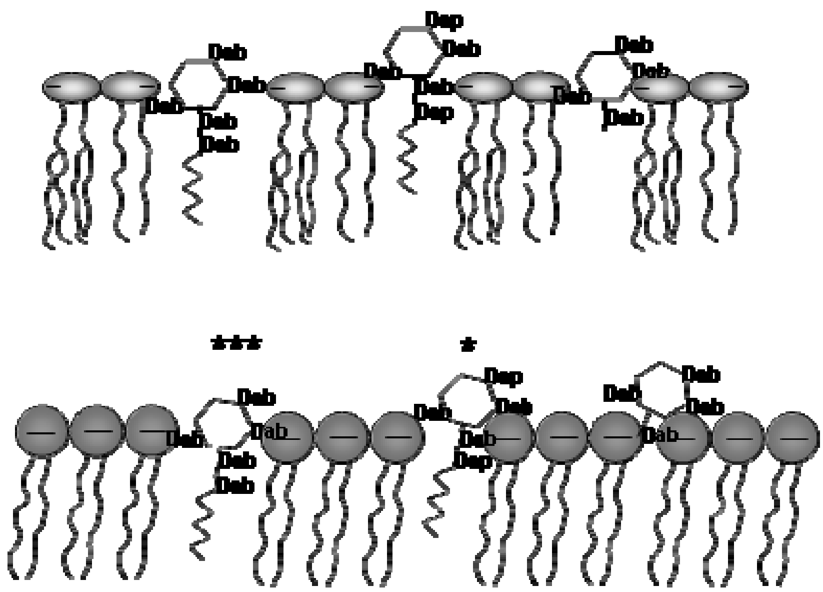

Figure 7. Cartoon schematic of possible interactions of the peptides with bacterial membranes. (*Top*) Lipopolysaccharide as OM model; (*Bottom*) Anionic phospholipid as IM model. Peptides represent (from left to right): PxB or sp-B; sp-D; PxB-np. Active forms are indicated with asterisks [39].

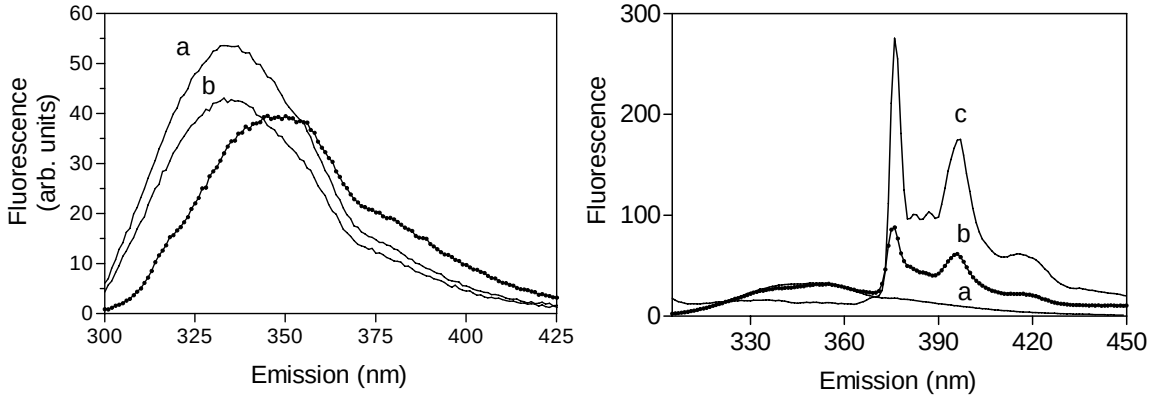

Figure 8. (Left) Tryptophan fluorescence emission spectra of sP-Bw in 10 mM Tris buffer (dotted line) or bound to vesicles of (a) POPG; (b) POPA. (Right) Self-association of sP-B bound to vesicles of POPG detected by intermolecular Trp-pyrene FRET. (a) sP-Bw; (b) mixture of sP-Bw and sP-Bpy; (c) same with POPG vesicles; peptide mol fraction 1% [41].

5. Conclusion

Naturally occurring polymyxins are cationic antimicrobial peptides highly effective against Gram-negative bacteria. The emergence of multiply antibiotic-resistant Gram-negative bacilli, frequently susceptible only to polymyxins, has sparked a renewed interest in these agents, and many efforts are directed towards the generation of new molecules with improved therapeutic index. For this purpose, a detailed knowledge on the mechanism of action of polymyxin and polymyxin derivatives in the membrane is fundamental. In our group we have studied the interaction of polymyxin B (PxB) with lipid vesicles and monolayers prepared with a lipid composition that closely mimics the bacterial inner and outer membranes. Results are consistent with a mechanism of action based on the simultaneous interaction with the two membranes and alteration of the membrane composition by PxB-induced membrane-membrane contacts and lipid exchange. Synthetic PxB-analogues have been specifically designed to get more insight into the mechanism of action at the molecular level. Analogs with intrinsic fluorescence indicate that peptide self-association in the membrane is necessary for intermembrane contact formation, whereas analogs with different modifications in the hydrophobic and polar residues reveal that insertion and membrane activity depends on the lipid composition and is the result of a combination of electrostatic and hydrophobic interactions. Synthetic analogues that act by the same mechanism than the parent peptide are promising molecules in the search for new antibiotics to treat resistant bacteria.

Acknowledgements

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5. New strategies in the modulation of fatty acid oxidation as a treatment for obesity

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Abstract. Strategies that enhance fat degradation or reduce caloric food intake could be considered therapeutic interventions to reduce not only obesity, but also its associated disorders. The enzyme carnitine palmitoyltransferase 1 (CPT1) is the critical rate-determining regulator of fatty acid oxidation (FAO) and might play a key role in increasing energy expenditure and controlling food intake. Our group has shown that mice overexpressing CPT1 in liver are protected from weight gain, the development of obesity and insulin resistance. Regarding food intake control, we observed that the pharmacological inhibition of CPT1 in rat hypothalamus decreased food intake and body weight. This suggests that modulation of CPT1 activity and the oxidation of fatty acids in various tissues can be crucial for the potential treatment of obesity and associated pathologies.

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Introduction

Obesity and its associated metabolic disorders, such as insulin resistance, type 2 diabetes, cardiovascular disease and other severe pathologies, are one of the most serious public health concerns of the twenty-first century. There are more than 500 million obese people worldwide and, more importantly, overweight and obesity are the fifth leading risk for global deaths [1]. In the last few years, a concerted effort has been made to understand the pathophysiology of obesity and particularly its association with insulin resistance. Various mechanisms have emerged in the past two decades to elucidate this causal relationship. i) Ectopic fat deposition: during obesity the capacity of adipose tissue to expand in order to store excess fat fails, resulting in lipid spillover to peripheral organs such as liver, skeletal muscle and pancreas [2,3]. This incorrect lipid accumulation creates a lipotoxic environment, mainly mediated by diacylglycerols (DAG), that blocks correct glucose transport and insulin signaling [4]. ii) Inflammation: the excess of lipids accumulated in the adipose tissue during obesity causes adipocyte hypoxia [5], endoplasmic reticulum (ER) stress [6], cell death, and finally FA spillover [4]. These events lead to the recruitment and activation of immune cells in the adipose tissue. Obese adipocytes and infiltrated immune cells secrete many inflammatory cytokines that promote a proinflammatory state that contributes to local and systemic insulin resistance [7,8]. iii) Food intake: the central nervous system, specifically the hypothalamus, is extremely important in obesity-induced pathologies, since it plays a major role in the control of food intake and the regulation of body weight. In fact, leptin, an adipocyte-secreted hormone, acts on the hypothalamus to inhibit food intake and control body weight and is essential in the interaction between the brain and other organs in obesity-related disorders [9,10].

Every therapeutic strategy to combat obesity is focused on increasing energy expenditure through regular exercise and/or reducing energy intake. However, the maintenance of lifestyle modifications for long periods is difficult and challenging. All the anti-obesity drugs on the market approach obesity by aiming to limit energy intake. Nonetheless, the list of food intake-limiting drugs withdrawn from the market for safety reasons seems to be constantly increasing: fenfluramine, desfenfluramine, sibutramine and rimonabant [11]. Currently, only orlistat and lorcaserin have an approved clinical indication for obesity treatment, but not worldwide, since the EMA is still studying the approval of the latter [12]. Owing to the pandemic of obesity-related diseases, there is a need for a wider range of drugs with different mechanisms and a safer profile to optimize and individualize obesity management therapies. The development of different strategies, such as those designed to increase lipid mobilization and oxidation, has become an imperative.

Long chain fatty acid oxidation plays a key role in the development of obesity and occurs in mitochondria. Lipid transport into the mitochondria is mediated by the carnitine palmitoyltransferase (CPT) system [13], which is comprised of CPT1, acylcarnitine translocase and CPT2. The first protein, CPT1, catalyzes the rate-limiting step in mitochondrial fatty acid oxidation (FAO), since it can be regulated by changes in malonyl-CoA levels. These levels are controlled by acetyl-CoA carboxylase (ACC), which controls malonyl-CoA synthesis, and malonyl-CoA decarboxylase (MCD), which catalyzes malonyl-CoA degradation [13]. Together, these components act as a metabolic network that senses the cell's energy state. Once long chain fatty acids have been degraded to acetyl-CoA in the mitochondria, they are transformed into ATP through the Krebs cycle and oxidative phosphorylation, in which ATP molecules are made up from the derived NADH and $FADH_2$ energy used to create the electrochemical gradient needed by ATP synthase [14].

Mammal tissues express three isoforms of CPT1: CPT1A, which was originally discovered in liver but is present almost ubiquitously [15], CPT1B that is present in muscle and heart [16], and CPT1C, the latest isoform to be discovered, which is expressed mainly in the brain [17].

Our research indicates that the modulation of mitochondrial bioenergetics, and especially FAO, is a good target for anti-obesity therapy. Our strategy is based on two main interventions: one in peripheral tissues such as muscle and liver, and the other at central level in the hypothalamus. In peripheral tissues, overexpression of the CPT1A gene seemed to be a suitable approach, because presumably the effects of this intervention would reduce the intracellular lipid content, and lead to a general improvement in cellular metabolism. Furthermore, we have produced a M593S CPT1A cDNA rat mutant (CPT1AM), whose translated protein is active, but totally insensitive to malonyl-CoA inhibition [18]. Therefore, overexpression of CPT1AM would disconnect glucose metabolism, which increases malonyl-CoA levels, from accelerated fatty acid catalysis. Thus, the reduction in cellular lipids would be produced much more efficiently. At central level, our approach is based on the inhibition of CPT1 activity, which is involved in the signaling pathway that controls food intake.

1. The enhancement of mitochondrial FAO in liver ameliorates insulin resistance and prevents obesity induced by a high-fat diet

In liver, conditions associated with prolonged energy excess or impaired FA metabolism lead to the accumulation of considerable amounts of lipids. This triggers the development of non-alcoholic fatty liver disease (NAFLD),

which involves a series of liver abnormalities. NAFLD produces an abnormal accumulation of lipids and inflammation. It also renders insulin unable to control hepatic gluconeogenesis. This is usually the beginning of diabetes, but episodes of steatohepatitis, cirrhosis and hepatocellular carcinoma are common at later stages of development [19-21].

Considering that NAFLD is ultimately the result of an imbalance between lipid inputs and outputs in the hepatocytes, any intervention that stimulates hepatic FAO should result in reduced hepatic fat accumulation. Several pharmacological approaches that activate FAO have been reported using PPAR [20,22] and AMPK [23,24] agonists and ACC antagonists [25,26]. More interesting are studies in rodents to decrease steatosis by increasing FAO specifically in liver. In these studies, short-term modulation of ACC [27] and MCD [28] gene expression produced a reduction in malonyl-CoA levels and an increase in FAO. In addition, a decrease in hepatic TAG content and insulin resistance was observed in obese animals. However, the notion that these target genes are implicated in other metabolic pathways raises the question of their effectiveness in long-term treatments.

Taking into account that the main factor that increases FAO is the mitochondrial membrane enzyme CPT1A, which is the critical rate-determining regulator of beta-oxidation, overexpression of the CPT1A gene seemed to be a suitable approach, because presumably the effects of this intervention would reduce the lipid content, and lead to a general improvement in hepatic metabolism. Hepatic overexpression of CPT1A mediated by adenovirus has been performed by Stefanovic-Racic *et al.* [29] in obese rats. They observed a slight increase in FAO that led to a reduction in hepatic TAG levels, but did not reveal any improvement in insulin sensitivity. The moderate effect might be due to the increased malonyl-CoA levels induced by a high-fat diet (HFD), which could limit *in vivo* CPT1A activity, in spite of the CPT1A overexpression.

To avoid malonyl-CoA inhibition, we generated a mutant CPT1A isoform, CPT1AM [18], that is insensitive to malonyl-CoA. Results published by our group confirm in the pancreatic cell line INS-1 [30] and muscle cell line L6E9 [31] that adenovirus-mediated overexpression of CPT1AM is more efficient at increasing mitochondrial FAO. Furthermore, overexpression of CPT1AM in L6E9 cells produces a 2-fold increase in palmitate oxidation, and decreases its esterification into cellular lipids. Similar results were obtained in hepatocyte primary culture by [32] and our group (Fig. 1). Adenovirus-mediated CPT1AM overexpression produced higher oleate oxidation than the CPT1A wild type isoform and a higher reduction in intracellular TAG content when hepatocytes where incubated in the presence of palmitate (Fig. 1).

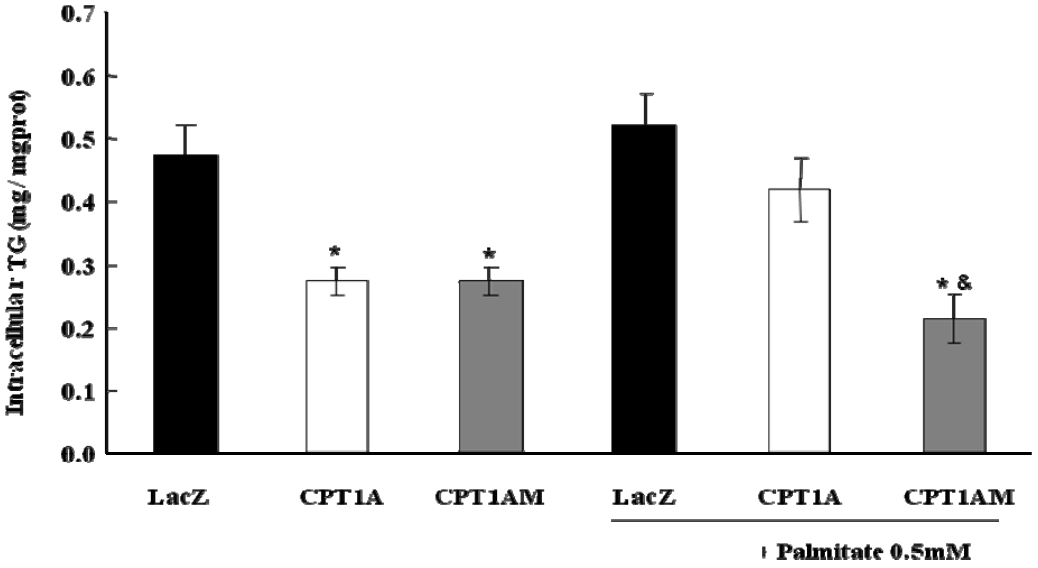

Figure 1. CPT1 overexpression reduced intracellular TAG content. Rat hepatocytes were transduced for 24 h. After this time, cells were treated with palmitate for 4 h. Lipids were extracted and TAG were measured using the Cromatest Triglyceride kit. $^{*}P < 0.05$ vs. Lac Z – Palmitate, $^{\&}P < 0.05$ vs. Lac Z + Palmitate.

In addition to *in vitro* studies, we performed *in vivo* experiments in mice fed with HFD [33]. We achieved hepatic gene transfer of CPT1A and the permanent active form CPT1AM to obese mice by injecting adeno-associated viruses (AAV) into the tail vein. The use of AAV-CPT1A and AAV-CPT1AM led to long-term liver-selective gene transfer that allowed us to evaluate the metabolic impact and underlying mechanisms of increased FAO in diet-induced and genetically obese mice. In these studies, we observed that HFD CPT1A- and CPT1AM-expressing mice showed a general improvement in hepatic glucose and lipid metabolism as a consequence of increased hepatic fatty acid flux through mitochondria. In turn, this prevented intracellular lipid accumulation in liver (Fig. 2) and ROS production and rescued the impaired hepatic insulin signal, especially in CPT1AM-expressing mice (Fig. 3). It seems that liver can deal with an increased flux of FA into the mitochondria, thus escaping from liver injury. This is due to the ability of liver to flip the balance from complete oxidation to ketone body production [34]. The ketone bodies produced by enhanced FAO are easily consumed by other tissues. This increases the flux of carbons from liver to other organs (Fig. 3). In addition, enhanced mitochondrial FAO in liver reduced the lipid accumulation in white adipose tissue and prevented the increase of body weight in these HFD mice. The impaired insulin signaling in tissues such as muscle and white adipose tissue was also ameliorated. These results have been further confirmed by Monsenego *et al.* [35].

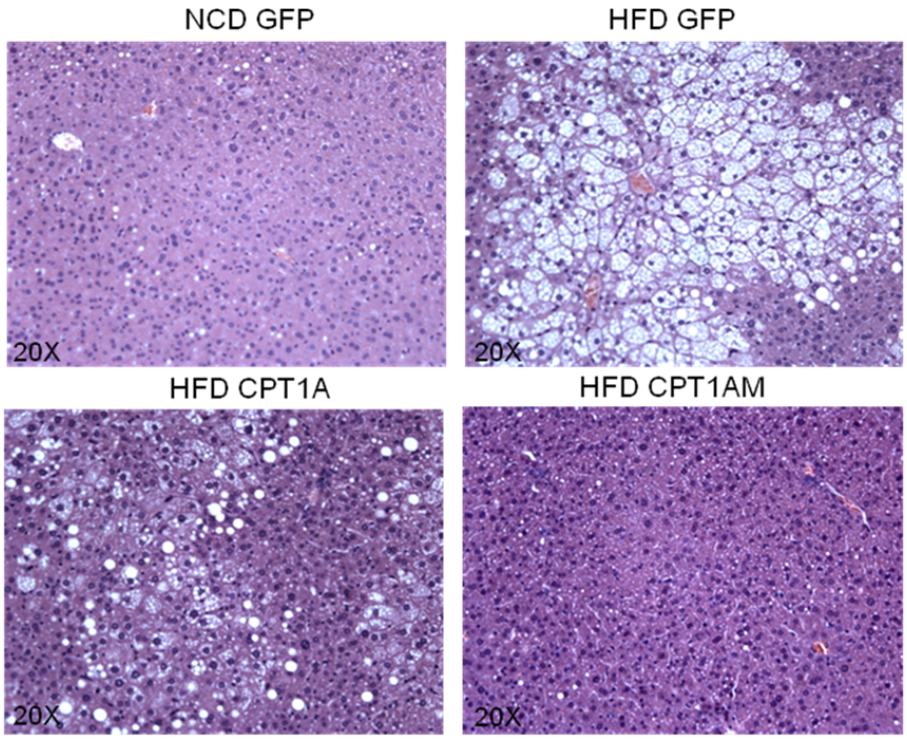

Figure 2. Liver histology in GFP, CPT1A- and CPT1AM-expressing mice. Liver histological sections (hematoxylin and eosin staining) from representative 29-week-old GFP control mice and GFP-, CPT1A- and CPT1AM-expressing HFD littermates.

In genetically obese *db/db* mice expressing CPT1AM we also observed an improvement in the hyperglycemia and hyperinsulinemia that these animals suffer. Taken together, these results highlight an increase in hepatic CPT1AM as a new strategy for the treatment of NAFLD/NASH pathologies and obesity (Fig. 3).

Another strategy has been proposed to improve mainly glucose homeostasis in HFD-treated mice. This approach is based on the administration of CPT1 inhibitors. Although this treatment reduced hepatic gluconeogenesis, it also caused hepatic steatosis in HFD-treated mice [36,37]. This side-effect has discarded the development of other CPT1 systemic inhibitors, such as etomoxir and 2-tetradecylglycidic acid, as a therapeutic tool. Taken together, these data support the idea that any strategy that can switch liver FA's fate from esterification towards oxidation has a beneficial effect on liver and on the whole body.

Recently, a new hepatic factor, fibroblast growth factor 21 (FGF21), has emerged as a key regulator in FAO and ketogenic activation. FGF21 is upregulated in liver during fasting [38,39] and has been increasingly indicated as a potential therapeutic agent in obesity-induced insulin-resistant states [40].

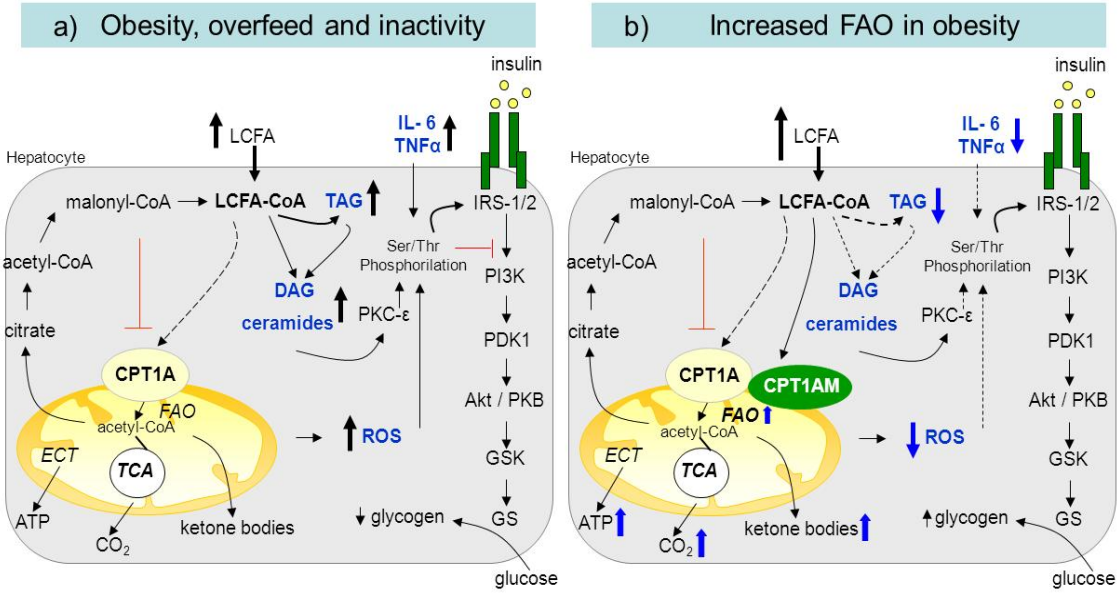

Figure 3. Effects of enhanced FAO in fatty liver. (a) Obesity increases fatty acid (FA) uptake, TAG (triacylglycerides), DAG (diacylglycerol), ceramides and other lipid derivatives that may inhibit insulin signaling. FA accumulation induces mitochondrial dysfunction and increased ROS production, oxidative stress and inflammation that could also disrupt insulin signaling. (b) Enhancing FAO by the overexpression of CPT1AM increases the production of ketone bodies, ATP and CO_2. The reduction of lipid content reestablishes lipid metabolism and insulin signaling. It also decreases inflammation and ROS production. FAO: fatty acid oxidation; GSK: glycogen synthase kinase-3; GS: glycogen synthase; IRS-1/2: insulin receptor substrate; LCFA: long chain fatty acids; PKC: protein kinase C; PI3K: phosphoinositide 3-kinase; PDK1: phosphoinositide-dependent kinase-1; PKB: protein kinase B; ROS: reactive oxygen species; TCA: tricarboxylic acid cycle; TNFα: tumor necrosis factor; NCD normal control diet.

2. Inhibition of CPT1 in the hypothalamus reduces food intake and body weight

The central nervous system (CNS) plays a major role in the evaluation and control of energy homeostasis. Blood concentrations of glucose and fatty acids are sensed by neurons of the hypothalamus, which adjusts feeding behavior by altering the expression of specific neuropeptides and neurotransmitters. It is now well established that hypothalamic lipid metabolism participates in this action of the hypothalamus and is linked to molecular mechanisms by which hormones and nutrients exert their central effect on food intake [41]. Recently, much effort has been invested in designing new anti-obesity drugs and modulating fatty acid metabolism to inhibit food intake.

Both malonyl-CoA, the first intermediate in *de novo* lipogenesis, and LCFA-CoAs have been proposed as satiety molecular signals in the hypothalamus [42-45]. For this reason, drugs designed to raise hypothalamic concentrations of these molecules are good candidates for the treatment of food intake disorders. The enzyme fatty acid synthase (FAS) catalyzes the synthesis of palmitate from acetyl-CoA and malonyl-CoA. It has been reported that FAS inhibition in the hypothalamus suppresses food intake through the accumulation of malonyl-CoA [46,47]. C75 is a synthetic inhibitor of FAS and has been proposed as an anti-obesity agent, since its administration decreases appetite and body weight in rodents [48]. However, the exact molecular mechanism underlying C75-derived anorexia is not completely understood. Firstly, some evidence showed a disconnection between C75-induced hypophagia and FAS inhibition in the hypothalamus [49]. Secondly, the inhibition of FAS by C75 produces an accumulation of malonyl-CoA that is difficult to reconcile with the activation of CPT1 reported by others authors [50,51]. Finally, our group previously reported that the Coenzyme A adduct of C75 (C75-CoA) is a potent inhibitor of CPT1 [52] (Fig. 4). Taking these data into account, further research is needed to clarify whether C75-induced hypophagia is directly related to hypothalamic inhibition of FAS, CPT1 or both enzymes.

It has been stated that pharmacological and genetic inhibition of CPT1 in the hypothalamus reduces food intake [53,54]. Rossetti and co-workers demonstrated that genetic inhibition of the isoform CPT1A in the mediobasal hypothalamus not only reduced feeding in rats, but also diminished hepatic gluconeogenesis. Similar results were obtained with tetradecylglycidic acid

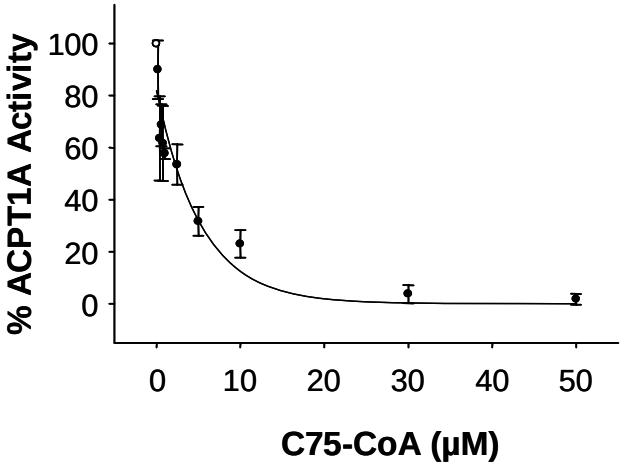

Figure 4. Effect of C75-CoA on the activity of yeast-expressed CPT1A. Mitochondrial extracts from yeast expressing CPT1A were preincubated with increasing concentrations of C75-CoA. CPT1A activity was measured, and data are expressed relative to control values in the absence of C75-CoA (100%).

and the compound ST1326, a synthetic CPT1 inhibitor. The observed anorexia was accompanied by a significant increase of LCFA-CoAs in the hypothalamus. The same authors demonstrated that central injection of oleic acid produced a reduction in food consumption, due to the downregulation of orexigenic neuropeptides in the hypothalamus [44]. Based on these results, our group attempted to explain the anorectic effects of C75 in terms of its inhibitory action on hypothalamic CPT1. We first demonstrated that, following its central administration, C75 is converted to C75-CoA in the hypothalamus [55]. In addition, central C75 administration produced a significant inhibition of CPT1 activity in the hypothalamus [55] (Fig. 5a). Furthermore, central injection of etomoxir, a well-known CPT1 inhibitor, also decreased feeding and reduced body weight in rats (Fig. 5b and c). Together, these data suggested that hypothalamic CPT1 inhibition could, at least partly, mediate the anorectic effect of C75.

We propose that the direct *in vivo* effect of C75-CoA on hypothalamic CPT1 explains the inhibition of CPT1 activity and the subsequent reduction in food intake. However, FAS could also be inhibited by C75 in the hypothalamus. Therefore, malonyl-CoA could be in excess and inhibit CPT1, together with C75-CoA (Fig. 6).

Despite the aforementioned results, further research is needed to unravel the exact mechanism by which CPT1 inhibition affects the expression of hypothalamic neuropeptides and, consequently, feeding behavior. Our group is currently working on two main research lines: the clarification of the exact role of hypothalamic CPT1 in the regulation of appetite and C75-derived

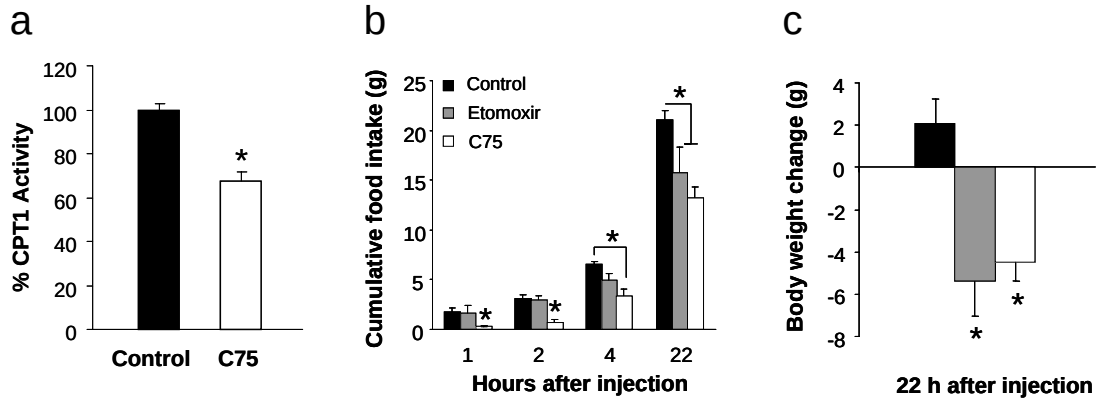

Figure 5. Central nervous system administration of C75 inhibits CPT1 activity and decreases food intake and body weight. (a) CPT1 activity after intracerebroventricular (i.c.v.) injection of C75 (control animals, RPMI medium). (b) Food intake measured in rats at 1, 2, 4 and 22 h after i.c.v. injection of C75, etomoxir and control (RPMI medium). (c) Body weight measured in rats at 22 h after i.c.v. injection of C75, etomoxir and control (RPMI medium). * $P < 0.05$.

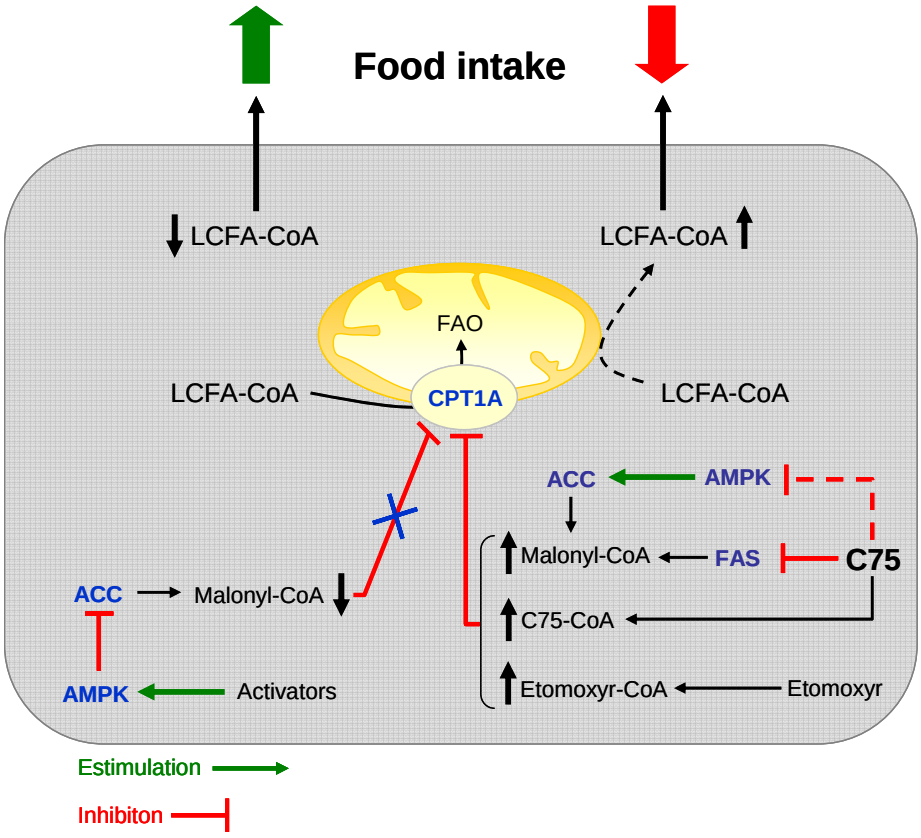

Figure 6. Model of action of C75 on hypothalamic fatty acid metabolism. The energy balance is monitored by the hypothalamus. The accumulation of malonyl-CoA due to FAS-inhibition by C75 leads to a reduction in CPT1 activity. This would increase the cytosolic pool of LCFA-CoA and thus decrease food intake. In addition, C75 may inhibit AMPK, resulting in activation of ACC and a consequent increase in malonyl-CoA levels [56]. Here, we have shown that C75 is converted into C75-CoA in the hypothalamus, which contributes to the direct inhibition of CPT1. Our experiments also demonstrate that other CPT1 inhibitors such as etomoxir produce similar effects of decreasing food intake. However, AMPK-activators lead to ACC inactivation, which reduces malonyl-CoA levels and CPT1 inhibition. Thus, increased FAO reduces LCFA-CoAs and promotes food intake.

anorexia, and the design and synthesis of new CPT1 inhibitors as a first step in the development of new anti-obesity drugs.

3. Conclusion

There is general agreement that we need to look for new treatments to fight against the current epidemic of obesity and related diseases. Dietary modification and regular exercise are two classical and very effective strategies for decreasing nutrient overload. However, long-term maintenance of these strategies is difficult and challenging. Thus, new therapeutic strategies are

needed to reduce the lipid burden and increase energy expenditure. Alternatively, new drugs should be developed to modulate eating behavior.

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6. Biotechnological production of taxanes: A molecular approach

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Abstract. Plant cell cultures constitute a promise for the production of a high number of phytochemicals, although the majority of bioprocesses that have been developed so far have not resulted commercially successful. An overview indicates that most of the research carried out until now is of the empirical type. For this reason, there is a need for a rational approach to the molecular and cellular basis of metabolic pathways and their regulation in order to stimulate future advances.

The empirical investigations are based on the optimization of the culture system, exclusively considering input factors such as the selection of cellular lines, type and parameters of culture, bioreactor design and elicitor addition, and output factors such as cellular growth, the uptake system of nutrients, production and yield. In a rational approach towards the elucidation of taxol and related taxane production, our group has studied the relationship between the taxane profile and production and the expression of genes codifying for enzymes that participate in early, intermediate and late steps of their biosynthesis in elicited *Taxus* spp cell cultures. Our results show that

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elicitors induce a dramatic reprogramming of gene expression in *Taxus* cell cultures, which likely accounts for the enhanced production of taxol and related taxanes and we have also determined some genes that control the main flux limiting steps. The application of metabolic engineering techniques for the production of taxol and taxanes of interest is also discussed.

Introduction

One of the most important characteristics of plants is their ability to form compounds that, though not essential for completing the plant's life cycle, are extremely useful for their adaptation to the environment. Many of these secondary compounds are considered of high value by the chemical and pharmaceutical industry due to their therapeutic and adaptogenic properties. It is estimated that 50% of drugs used in the preparation of medicines are natural products of plant secondary metabolism. Therefore, having been used as drugs since ancient times, by all the cultures ever to exist on our planet, plants remain an inexhaustible source of new medicines [1].

The natural exploitation of medicinal plants by means of their culture and extraction of their main active elements is fraught with geographical, environmental, economical and political risks and difficulties. So it is hardly surprising that one of the main objectives of plant biotechnology is the large-scale production of secondary compounds with industrial applications. The biotechnological production of secondary metabolites using different *in vitro* techniques has been the focus of considerable research in recent years. Besides their use as pharmaceuticals, fragrances, colorants, aromatics and pesticides, secondary metabolites have recently been recognized as important disease-preventative ingredients in food, resulting in the term nutraceuticals [2].

The advantages of this technological approach over conventional agriculture include: independence from geographical and seasonal variations and environmental factors as well as from political interference; a defined production system that ensures a continuous supply of products of uniform quality and quantity; the possibility of obtaining new compounds not originally formed in the cultivated plant; an efficient recovery of the product of interest; an extremely fast production and also stereo- and regiospecific biotransformations from inexpensive precursors.

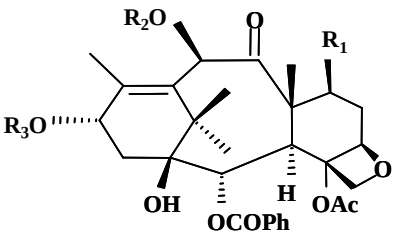
Although *in vitro* culture of plant cells and tissues is routinely used in agriculture for plant breeding and micropropagation, its application in the commercial production of pharmaceuticals and other secondary compounds of interest is limited to only a few procedures [3-5].

An essential requirement for the application of *in vitro* plant culture techniques in industrial-scale production of therapeutic compounds is that its cost is lower than culturing the intact plant or other procedures. Given the

complexity of these techniques, together with the costs involved in sterilization and use of bioreactors, the new technologies can only be assigned to the production of compounds of high added value.

Taxol (Fig. 1) is a secondary compound of great interest due to its wide spectrum of antineoplastic action. The main limitation to its therapeutic use is a limited supply from its natural source, the inner bark of *Taxus* species [6]. One of the most successful anticancer drugs to be developed in the past 50 years, taxol produced by Bristol-Myers Squibb (BMS) reached worldwide sales of $1.5 billion in 1999. Although this company subsequently reported a 24% decrease in taxol sales, from $422 millions in 2006 to $385 millions in 2007 [BMS 2008 Annual Report], this reduction is primarily due to patent expiry and increased generic competition in Europe, as well as generic entry in Japan during the third quarter of 2006 [1]. The total market for taxol remains well above $1 billion per year [www.strategyr.com/Bulk Paclitaxel Market Report.asp] and continues to expand, with new clinical uses anticipated [7].

To combat the patent expiries, supergeneric versions of taxol, such as Cell Therapeutics' Xyotax (polyglutamate paclitaxel) and Abraxis Oncology's Abraxane (nanoparticle albumin-bound paclitaxel) have been developed, offering significant advantages over taxol in terms of adverse effects and drug delivery.



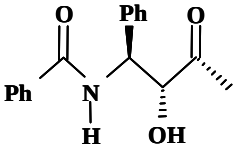
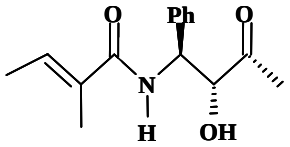
Taxol ®	**R_1 = OH; R_2 = Ac; R_3 =**	
Cephalomaninne	**R_1 = OH; R_2 = Ac; R_3 =**	
Baccatin III	**R_1 = OH; R_2 = Ac; R_3 = H**	
10-Deacetylbaccatin III	**R_1 = OH; R_2 = H; R_3 = H**	

Figure 1. General structure of taxol and related taxoids. Ac: acetyl group; Ph: phenyl group.

Sales of Abraxane rose to $275 millions in 2009 [www.BioPortafolio.com: emerging oncology treatments: a focus on targeted therapeutics, supergeneric reformulations and supportive care] reflecting the growing market for taxol and its derivatives. Consequently, taxol and its semisynthetic precursors, including baccatin III, 10-deacetylbaccatin III and cephalomaninne (Fig. 1), are considered to be high added value secondary compounds.

Plant cell cultures constitute a promise and almost a reality for the controlled production of a considerable number of secondary compounds of interest, including taxol and its derivatives, although most of the bioprocesses developed up to now have not turned out to be commercially viable. Much of the research in this field carried out during the last decades has been empirical, concentrating on optimizing the culture system via known in-put factors (cell line selection, culture media, culture conditions, elicitors, bioreactor culture, etc.) and out-put factors (growth, production, yield, etc.) However, the most successful strategies and technologies depend on a rational approach to the molecular bioprocesses of plant cells, based on the understanding of biosynthetic pathways and their regulation.

1. Empirical studies

Biotechnological production of taxol has been studied since the early 1990s. Early work carried out by different research teams [8-10] showed that calli and cell suspensions obtained from young stems of *Taxus* sp. were able to produce taxol at least to the same extent as the intact plant.

In order to increase the productivity of taxol and related taxoids in cell and tissue cultures, various strategies, including optimisation of culture conditions, selection of high-producing cell lines, use of elicitors, and addition of precursors, have been examined by many researchers [11-18]. There are excellent reviews about the biotechnological production of taxol and current knowledge of its metabolism in cell cultures [19-25].

Establishment of *Taxus* spp. cell cultures

In order to obtain cell suspensions, initially on a small scale and then at bioreactor level, it is first necessary to establish fast-growing callus cultures from which the cell suspensions are derived. We obtained *Taxus* callus cultures from young stems of 3-4 year-old yew trees cultured in optimum conditions [26]. In order to develop a biotechnological system for an efficient taxol production, we optimized the culture conditions by assaying several basic media, plant growth regulators, sugar supplements, etc. As secondary metabolite production in plant cell cultures does not usually depend on

growth, a two-stage culture system was established. Plant cells are first cultured in a medium optimized for their growth, which is then replaced by a production medium that principally stimulates the biosynthesis of secondary metabolites [27]. This system has the added advantage of permitting the addition of biosynthetic precursors and elicitors when the secondary metabolite production is at its highest, that is, during the culture's second stage. Taxol production was clearly enhanced by transferring cells from the optimum growth medium to the optimum medium for taxane production.

Elicitor treatments

A high increase in taxol and baccatin III production was observed in the MeJ-supplemented culture medium of different T. baccata cell lines (Fig. 2). We have also supplemented the culture medium with a variety of elicitors, finding methyl jasmonate (MeJ) and coronatine (Cor) to be the most effective in promoting taxane biosynthesis and accumulation [27-31]. When comparing the maximum levels of taxane production in *T. media* cell cultures,

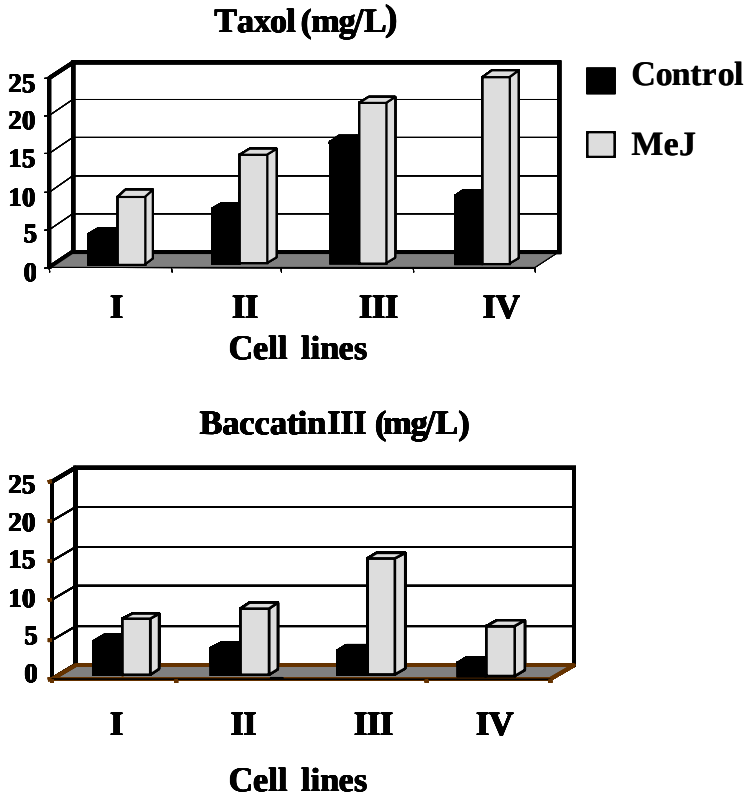


Figure 2. Taxol and baccatin III production in four different *T. baccata* cell lines treated with methyl jasmonate (MeJ, 100 μM) after 21 days of culture in the optimum medium for production.

we observed that the content of baccatin III in the Cor-treated cultures was 21.6- and 4.8-fold higher than in the control and MeJ-treated cultures, respectively, whereas MeJ elicitation increased baccatin III production 4.5-fold compared to the control. Taxol production under Cor treatment was 3.6- and 9.0-fold greater than in MeJ-treated and control cultures, respectively (Fig. 3) [31].

A notably high level of baccatin III accumulated in the Cor-treated cultures (52 mg/L) at day 16, when the productivity rate reached 3.3 mg L^{-1} day^{-1}. This is a very promising result, since new taxol-related compounds with improved efficacy and less toxicity are currently being sought, and most of them are being obtained semisynthetically from the natural precursor baccatin III [25].

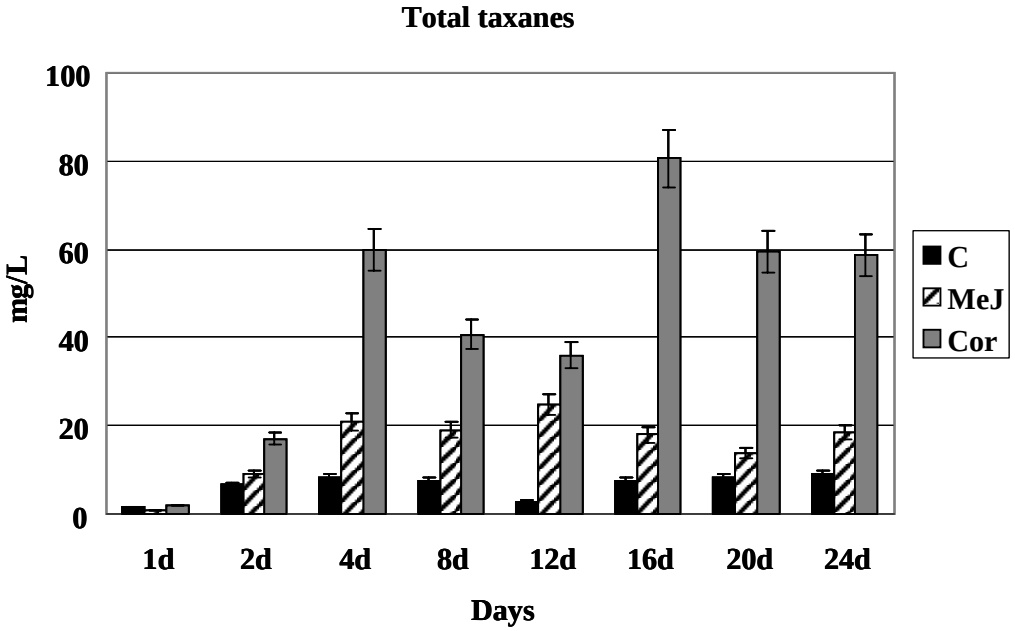


Figure 3. Total taxane content (cell associated + extracellular) in the TXS cell line growing for 24 days in the production medium without (Control, C) and with 100 μM methyl jasmonate (MeJ) or 1 μM coronatine (Cor). Data are average values from three replicates ± SD.

Scale-up to bioreactor culture

After optimizing the culture conditions, as well as environmental and physical factors, the next step for the profitable production of valuable secondary compounds is to scale up the culture. Bioreactors are used since they can be applied in large-scale production and allow a close control of culture conditions. The production of taxol and related taxanes in a variety of bioreactors has been described by several researchers [24, 32-34]. We compared the taxane levels achieved by *T. media* cell cultures grown in

Erlenmeyer flasks and a stirred bioreactor, in both cases using the same cell line and production medium supplemented with MeJ. Scaling up was found to improve the production of taxol and baccatin III 2.4- and 9 –fold, respectively (Fig. 4) [33].

Numerous studies have also reported that the immobilization of plant cell suspensions enhances the production of valuable plant metabolites [35-37]. This increase is probably due to the higher cell concentration, better cell-cell contact and more favorable conditions for cell differentiation.

In our case, taxol production clearly improved when cell suspensions were immobilized in 2% alginate beads and cultured in a bioreactor, using the optimum MeJ-supplemented medium for taxol biosynthesis. When taxol production was at its highest, at day 16, its levels were more than 5 times higher than those obtained by the same cell line growing freely in the same conditions (Fig. 5) [33].

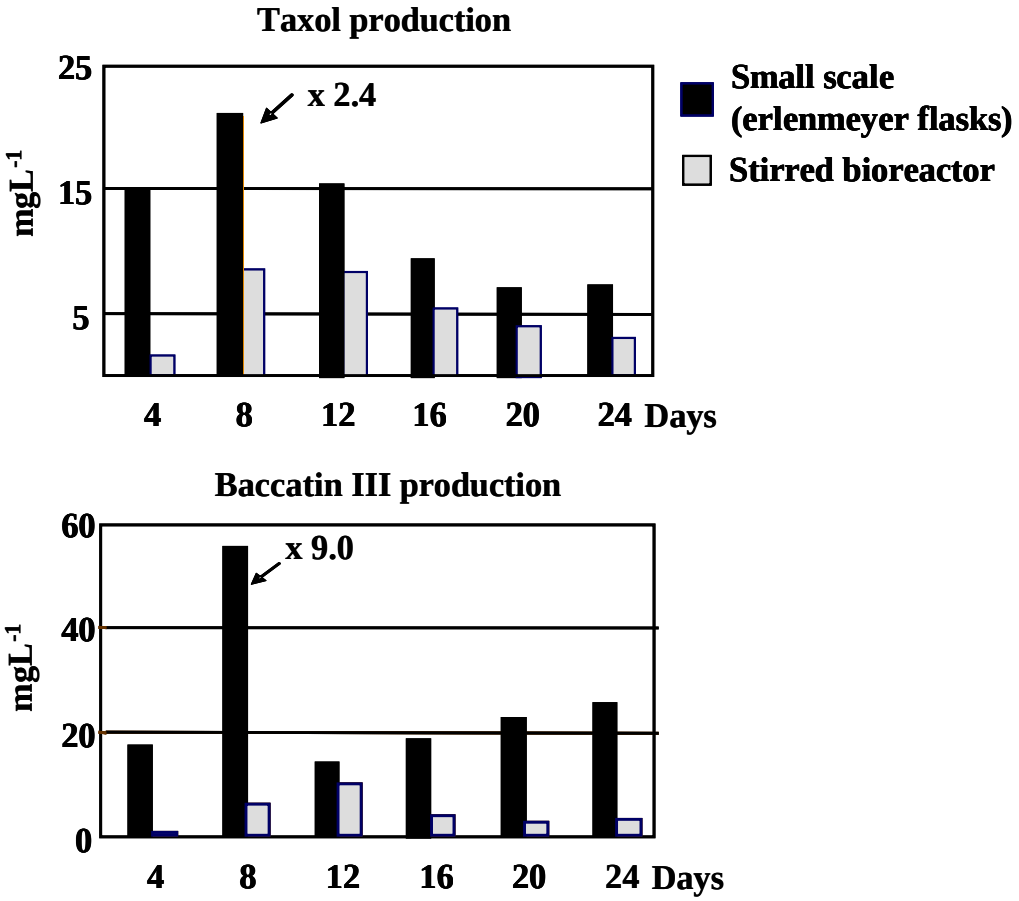


Figure 4. Total content (cell-associated + extracellular) of taxol and baccatin III in small-scale *T. baccata* cell cultures (Erlenmeyer flasks) and in a 5 L stirred bioreactor. In both cases cells were grown for 24 days in the optimum medium for production supplemented with methyl jasmonate (100 μM).

Figure 5. Configuration of a 5 L air-lift bioreactor used to culture *T. baccata* immobilized cells.

2. Rational approach

In order to convert the potential of cell cultures into a commercial reality, after empirical optimization studies, a molecular approach is required, since it is necessary to know how the factors that improve production also affect molecular processes in producer cells, such as metabolic profiles, gene expression, enzymatic activity, etc.. These studies allow a better understanding of the biosynthetic pathways and their regulation.

The taxol biosynthetic pathway consists of 19 metabolic steps, 12 of which are known and have had their corresponding genes cloned and sequenced. The functions of the remaining 7 steps are understood, but the genes involved have not yet been sequenced [38-41].

In order to understand the limiting steps of the biosynthetic production of taxol, as well as its metabolic profile, it is necessary to compare the transcriptome of the known genes involved in taxol biosynthesis in both elicited and unelicited cultures [41-44].

Transcriptomic profiling

To investigate the elicitor mode of action in taxane biosynthesis and the relationship between gene expression and the pattern of taxane production, we used qRT-PCR to profile the expression of genes encoding enzymes involved in taxol biosynthesis in *T. media* cell cultures elicited with MeJ or Cor [31]. The studied genes and the enzymes they encode were: *TXS*

(taxadiene synthase) and *T13αOH* (taxadiene 13α-hydroxylase), both involved in early synthetic steps; *T7βOH* (taxane 7β-hydroxylase), *T2αOH* (taxane 2α-hydroxylase) and *DBAT* (10-deacetylbaccatin III-10β-*O*-acetyltransferase), which control intermediate synthetic steps; and *PAM* (phenylalanine aminomutase), *BAPT* (baccatin III-3-amino-13-phenylpropanoyltransferase) and *DBTNBT* (3'*N*-benzoyl transferase), which are involved in the last synthetic steps (Fig. 6).

Gene expression was determined from 1 h to 4 days after elicitation. Subsequent transcript accumulation is not shown in the figures because we observed that the highest expression and induction thereof occurs in this early period [42, 45]. Similar results have been reported by other research groups [41, 46].

The expression of the *TXS* gene, which controls the first committed step of the taxol biosynthetic pathway [47], was greatly enhanced by the presence of elicitors in the culture medium (Fig. 7). Maximum *TXS* transcript levels in the Cor-elicited cultures were 4.8-fold higher than in the control, observed at 24 h in both (Fig. 7). Although values were similar under both elicitors (140 and 130 times higher than the reference value) ($p<0.05$), it is notable that *TXS* transcript levels peaked 3 days earlier in Cor-treated cultures. We had previously

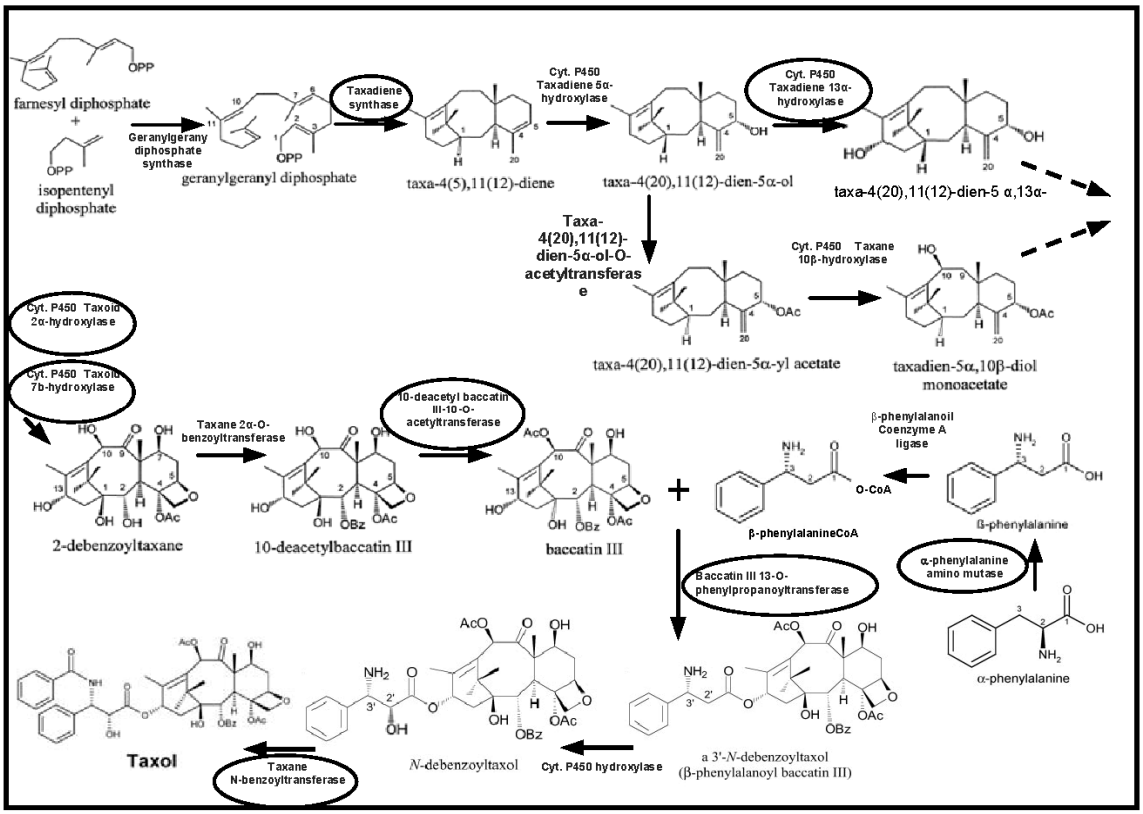


Figure 6. Taxol biosynthetic pathway. The enzymes controlling the steps discussed in the transcriptome study are highlighted with a circle.

shown that the *TXS* gene is highly induced in MeJ-elicited cell suspension cultures [42].

Taxadiene-13α-hydroxylase (T13αOH) is the enzyme that hydroxylates taxa-4(20),11(12)-dien-5α-ol to taxa-4(20),11(12)-dien-5α-13α-diol [48]. As shown in Fig. 7, significantly higher transcript accumulation of this gene was observed in Cor- compared with MeJ-treated cultures ($p>0.05$), and in both elicited cultures compared with the control ($p>0.05$). Results obtained by Nims *et al.* [41] indicate that *T13OH* expression increases during the first hours after MeJ treatment. In our case, although RNAm accumulation was evident after 1 h of elicitation, the highest level was achieved after 4 days. The difference is probably due to the use of different cell lines and culture systems.

It is known that there is a branch point after the biosynthesis of taxa-4(20),11(12)-dien-5α-ol, which can be the substrate either for an acetylation at C5OH and a subsequent hydroxylation at C10, or for an hydroxylation at C13 of the taxane skeleton, giving taxa-4(20),11(12)-dien-5α-acetoxy-10β-ol or taxa-4(20),11(12)-dien-5α-13α-diol, respectively (Fig. 6). Although we did not study the expression of genes *(TAT* and *T10βOH*) involved in the metabolic branch leading to taxa-4(20),11(12)-dien-5α-acetoxy-10β-ol, the high levels of the *T13OH* gene observed in our TXS cell line may suggest that, after elicitation with MeJ and Cor, taxol biosynthesis proceeds mainly through the step catalysed by the T13αOH enzyme. Nims *et al.* [41] also reported a preference for the T13αOH-side of the branch pathway in elicited *T. cuspidata* cell cultures, which confirms metabolic profiling data [49] showing that precursor flux leading to taxol is via 5α, 13α-diol through T13αOH, rather than 5α-yl-acetate derived from the alternative branch controlled by the TAT enzyme.

In the intermediate steps of taxol biosynthesis, the enzymes taxane-2α-hydroxylase (T2αOH) and taxane-7β-hydroxylase (T7βOH) catalyse the hydroxylation at the 2C and 7C positions of the taxane skeleton, respectively [50, 51] (Fig. 6). The two corresponding genes showed similar expression patterns, which were comparable to that of the *T13αOH* gene (Fig. 7).

The expression of the *DBAT* gene, corresponding to the enzyme responsible for the transformation of 10-deacetylbaccatin III into baccatin III (Fig. 7) [52], in MeJ-and Cor-treated cell cultures was already very high at 12 h, and continued increasing until days 1-2, decreasing thereafter to values similar to those observed at 12 h. The highest values observed in both cultures were 5 and 3 times higher, respectively, than in the control (Fig. 7).

The PAM gene, which encodes the enzyme responsible for the formation of β-phenylalanine from its isomer α-phenylalanine (Fig. 6) [53], is considered to be the first gene involved in the biosynthesis of the taxol lateral

chain. The expression of this gene in control cells remained very low throughout the 4 days of the study. In the elicited cultures (Fig. 7) two expression peaks were observed. The first occurred only 1 h after elicitation, with PAM transcript levels in the MeJ- and Cor-treated cultures being 43 and 63 times higher, respectively, than the reference value. The second peak, which was the maximum PAM transcript level achieved, was observed at day 4 under MeJ and day 2 under Cor treatment, being 52 and 93 times higher, respectively, than the reference value, and 29 and 53 times higher than in the control. It is notable that Cor was almost twice as effective as MeJ for the induction of the PAM gene.

In the final steps of the metabolic pathway (Fig. 6), the enzyme baccatin III 13-O-(3-amino-3-phenylpropanoyl) transferase (BAPT) [54] is responsible for binding the lateral chain to baccatin III, leading to taxanes with β-phenylalanine as a lateral chain. The enzyme N-benzoyl transferase (DBTNBT) [55] is involved in the last metabolic step leading to taxol. Control cultures grown in the production medium presented only a slight increase in the expression of the two corresponding genes over the 4 sampled days (Fig. 7). When comparing the elicitor treatments, the highest expression level of both genes was observed under MeJ, it being 1.3 times higher than the maximum under Cor. However, Cor caused an earlier activation, as shown in Fig. 7 (days 1-2 and 4, respectively).

Our results have shown that all the studied genes were induced to a variable extent by the presence of the elicitors MeJ (100 μM) and Cor (1 μM). The elicitors enhanced the expression of genes involved in the formation of the polyhydroxylated precursor of taxol and the phenylalanoyl CoA chain, but had a more limited effect on the last two steps of the taxol biosynthetic pathway (Figs. 6 and 7). The elicited cultures clearly showed different taxane patterns and gene expression profiles, suggesting differing action mechanisms, despite the structural similarity of Cor and the active jasmonate JA-Ile.

Our metabolic studies have demonstrated that the presence of Cor in the culture medium dramatically enhanced taxane production, particularly of baccatin III and to a lower extent taxol, the highest levels of both taxanes being 4.8- and 3.6-fold greater than in the MeJ-elicited cultures, and without affecting cell growth.

Taken as a whole, our results show that both MeJ and Cor induce a dramatic reprogramming of gene expression in *Taxus* cell cultures, which likely accounts for the enhanced production of taxol and related taxanes. Compared with MeJ, Cor elicitation did not result in a significantly higher induction of the studied taxane biosynthetic genes but peak expression levels were generally observed earlier. The resulting earlier availability of precursors could be responsible for the higher amount of total taxanes achieved in cultures elicited by Cor rather than MeJ (3.3-fold higher, on average).

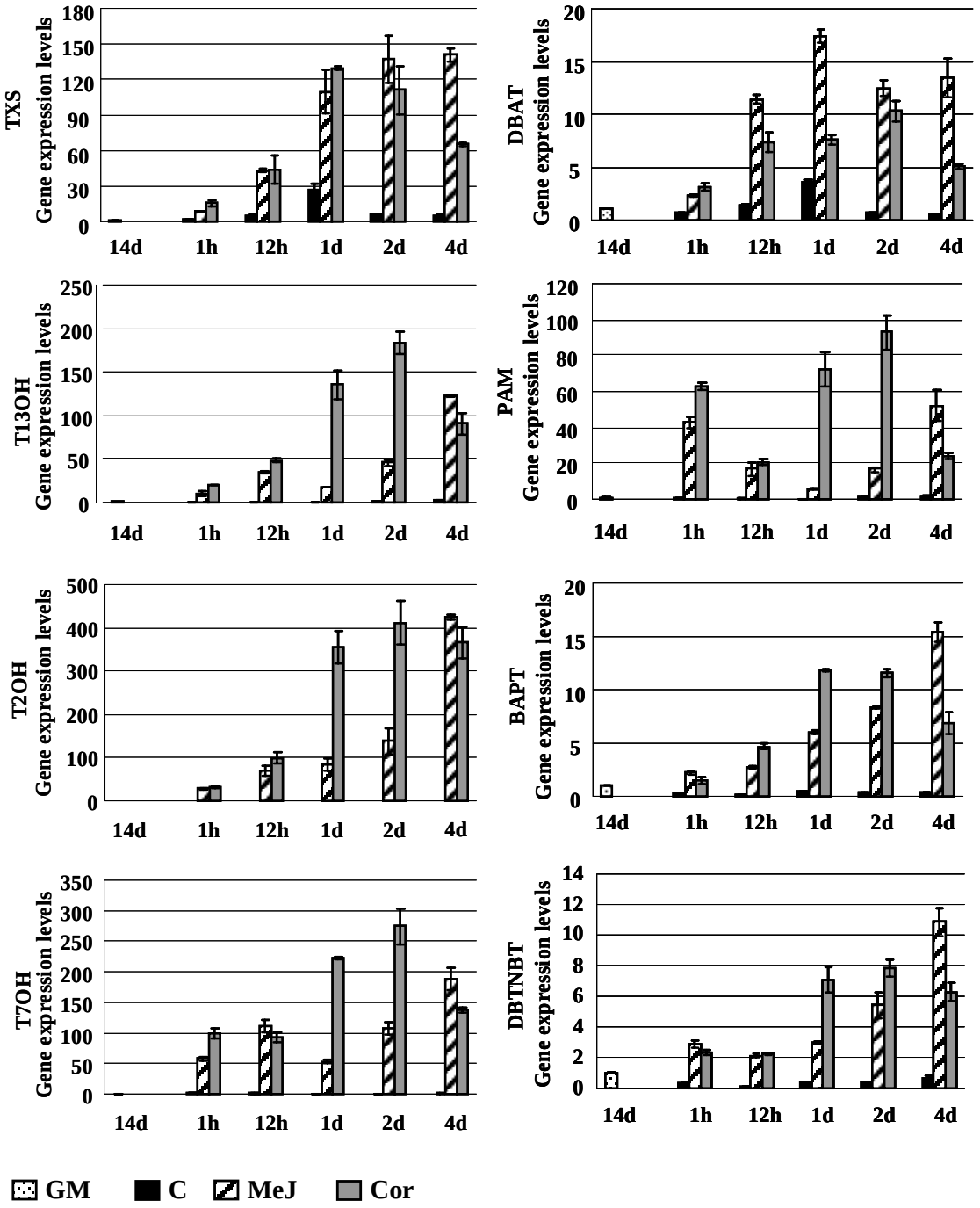


Figure 7. *TXS, T 13-OH, T 2-OH, T 7-OH, DBAT, PAM, BAPT* and *DBTNBT* gene expression in the TXS cell line cultured in the production media for 24 days under three different treatments: Control, 100 µM MeJ and 1 µM coronatine. GM: growth media; C: production media (control); Me: PM with MeJ; Co: PM with coronatine.

Determining the transcript profile of genes involved in taxol biosynthesis demonstrates that the presence of elicitors in the medium increases gene expression to varying degrees. The results also indicate that the transferases controlling the last steps of taxol biosynthesis probably also catalyze the

limiting steps, and consequently are ideal targets for metabolic engineering techniques in the enhancement of taxol production.

3. Metabolic engineering of *Taxus*

As *Taxus* species belong to the Gymnosperm class, they are very difficult to genetically transform. There have been many attempts to obtain transformed *Taxus* cell cultures using the *Agrobacterium* system or biolistic techniques, but with little success to date. In 1994, Han *et al.* [56] obtained gall cell lines after inoculating shoot segments of *T. baccata* and *T. brevifolia* with two strains of *A. tumefaciens*. In 2000, Furmanova and Syklowska-Baranek [57] established transformed root cultures of *T. x media* by directly infecting several kinds of explants with three strains of *A. rhizogenes*. In 2005, Ho *et al.* [58] described taxol production in MeJ-elicited *T. marei* cell cultures harboring the gene that encodes the enzyme 10-deacetyl baccatin III-10-O-actyl transferase (DBAT). More recently, Zang *et al.* [59], in *T. chinensis* cell cultures overexpressing the same gene, obtained a taxol production 1.7 times higher than that achieved by the untransformed cells. By using particle bombardment-mediated transformation, Vongpaseuth *et al.* [60] have successfully obtained a transient transformation of *Taxus* spp. with the aim of characterizing the genes involved in taxol biosynthesis and its regulation. Following the same transformation methodology, Li et al. [61] obtained *T. chinensis* cell cultures overexpressing a 9-*cis*-epoxycarotenoid dioxygenase gene, which encodes a key enzyme in the biosynthesis of abscisic acid (ABA), a phytohormone involved in plant responses to different types of stress. The taxol production in these transformed cell cultures was found to be 2.7-fold higher compared with untransformed cultures. However, apart from these studies, taxol production in transformed cell or root cultures has been scarcely explored.

We focused our attention on taxadiene synthase, the enzyme controlling the step that diverts GGPP from the metabolic pool to taxane biosynthesis, interested in gaining further insight into the key role it plays in taxane production overall. To this end, we established *T. media* cell cultures overexpressing the *txs* gene of *T. baccata*, but due to the difficulties of genetically transforming *Taxus* cell cultures with *A. tumefaciens,* we first obtained *T. media* transformed roots carrying this transgene (Fig. 8a), which were then dedifferentiated (Fig. 8b, c) [45]. Taxane production in the transformed cultures (Fig. 8d) was compared with an untransformed *T. media* cell line cultured in the same conditions. The highest taxane production was observed in the TXS cell line grown in the optimized production medium with MeJ (100 μM), it being 265% greater than in the untransformed control.

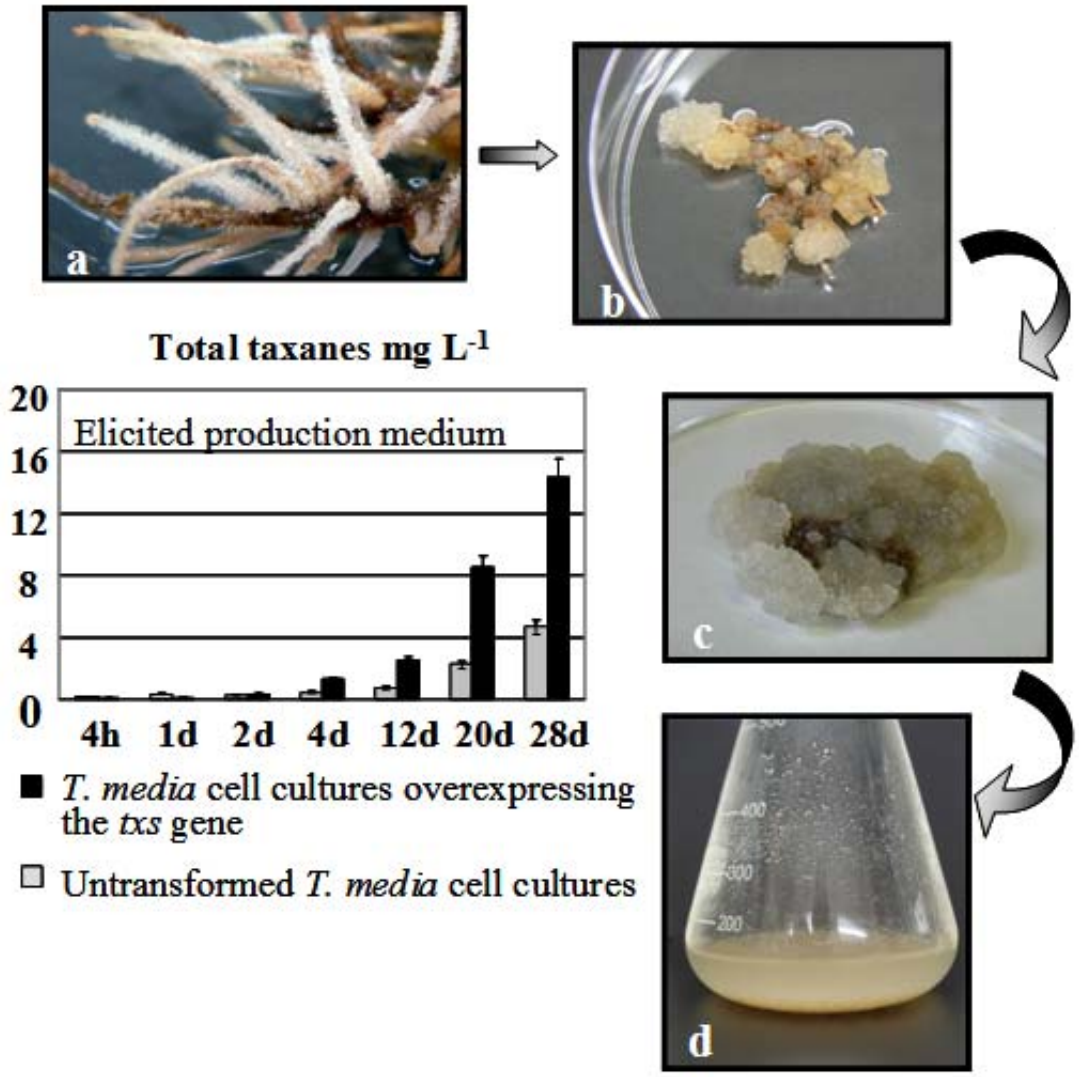


Figure 8. Steps followed to establish transformed cell cultures from transformed root cultures. Total taxane production in the *T. media* transformed cell cultures overexpressing the *txs* gene of *T. baccata*.

Notably, taxane levels as well as *txs* gene expression and TXS activity in both transformed and untransformed cell lines were clearly dependent on the elicitor action.

Knowledge of the genes involved in key steps of the taxol biosynthetic pathway and of how they are regulated, coupled with advanced metabolic engineering techniques, will allow transgenic cell cultures overexpressing target genes to be obtained, and will consequently lead to the establishment of highly productive cell cultures at an industrial level. We believe that our results could be useful for the development of rational strategies, including elicitation and metabolic engineering, to improve the yield of paclitaxel and the two related taxanes, 10-deacetylbaccatin III and baccatin III, which, as

previously mentioned, are used in the semisynthesis of the chemotherapeutic agent.

An aspect of taxane production that deserves highlighting is the mechanism of taxol excretion from cells, which could be enhanced by employing a two-phase culture system, so far not assayed in *T. baccata* cell suspensions. Future studies could combine an empirical and rational approach, assaying two-phase cultures to develop a biotechnological system for a high taxol production.

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7. Phylogenetic studies in Gnaphalieae (Compositae): The genera *Phagnalon* Cass. and *Aliella* Qaiser & Lack

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Abstract. The precise generic delimitation of *Aliella* and *Phagnalon*, and their closest relatives within the Gnaphalieae are discussed in this review. Among the main results obtained, we have found that the genera *Aliella* and *Phagnalon* are nested within the "*Relhania* clade" and *Anisothrix, Athrixia* and *Pentatrichia* are their closest relatives. *Macowania* is also part of the "*Relhania* clade", whereas the subtribal affinities of *Philyrophyllum* lie within the "crown radiation clade". The monophyly of *Aliella* and *Phagnalon* is not supported statistically. In addition, *Aliella* appears to be paraphylethic in most of the analyses performed. The resulting phylogeny suggests an African origin for the ancestor of *Aliella* and *Phagnalon* and identifies three main clades within *Phagnalon* that constitute the following natural groups on a geographic basis: (1) the Irano-Turanian clade; (2) the Mediterranean-Macaronesian clade; and (3) the Yemen-Ethiopian

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clade. Some endemics to Yemen and Ethiopia appeared merged in the Mediterranean-Macaronesian clade, providing new evidence of the phytogeographical links between Macaronesia, Eastern Africa and Southern Arabia. Incongruities between the chloroplast and nuclear molecular data and the lack of resolution in some clades may indicate that hybridization could have played an important role in the evolution and diversification of both *Phagnalon* and *Aliella*.

1. Historical overview, the Compositae family and the tribe Gnaphalieae

Aliella and *Phagnalon* belong to the Compositae family, which is the plant family with the largest number of described species, reaching 24,000–30,000 species distributed in 1.600–1.700 genera, with a cosmopolitan distribution except for Antarctica [1,2]. As it would be expected from its wide distribution, species of Compositae grow in a wide diversity of habitats: grasslands, wooded grasslands and montane vegetation, but they are comparatively scarce in humid tropical forests. In the same line, the morphological characters also exhibit wide variability. Species of Compositae exhibit most of the life forms, including annuals, pyrophytes, hemicryptophytes, trees, succulent plants, halophytes, lianas and also epiphytes and aquatics, although the majority of genera are subshrubs, shrubs or perennial herbs [3]. Another highly variable morphological trait is the number of florets per capitulum, which varies from one to 1.000 [1]. In spite of this high variability, the family has been traditionally considered a monophyletic group, morphologically well-defined by a combination of characters: (i) florets arranged on a receptacle, in centripetal heads surrounded by involucral bracts; (ii) syngenesious anthers, fused in a ring with the pollen pushed by the style; and (iii) fruits in achenes, usually with a pappus [2].

The tribe Gnaphalieae is one of the largest tribes of the Compositae, containing approximately 180–190 genera and 1,240 species. It is most diverse in the southern hemisphere, with three main centres of diversity: South America, South Africa, and Australia [4, 5, 6]. The current Gnaphalieae were initially classified under the Inuleae by Cassini [7] see Table 1. Later on, Bentham [8] split the Inuleae in nine subtribes based on the presence of homogamous or heterogamous capitula, and on other characters like the morphology of the receptacle, style and bracts. The majority of Gnaphalieae were included in the subtribes Filagineae, Gnaphalieae, Angianthieae, Relhanieae and Athrixieae. Merxmüller *et al.* [9] recognized three subtribes within the Inuleae: Inulinae, Gnaphaliinae and Athrixiinae, based on cytological, phytochemical, palinology and other traditionally used

morphological characters. These last authors placed the Gnaphalieae mainly in subtribe Gnaphaliinae, although some genera were also accommodated in the Athrixiinae. The Gnaphalieae were first recognized as independent from the Inuleae by Anderberg [4, 10], who divided the classic Inuleae [9] into three tribes: Inuleae, Plucheeae and Gnaphalieae. Anderberg [4] suggested classifying the Gnaphalieae into five subtribes (Angianthinae, Cassiniinae, Gnaphaliinae, Loricariinae and Relhaniinae) based on cladistic analyses of morphological characters, except for some genera that were left in what he called the "basal group". Several phylogenetic studies based on DNA sequences agreed in considering the Gnaphalieae as independent from the Inuleae [11, 12, 13].

Table 1. Major taxonomic treatments of Gnaphalieae according to Ward *et al.* [6]. Asterisks indicate that the genera included in that sections or tribes currently belong to the modern tribe Gnaphalieae.

Cassini (1822)	**Bentham (1873)**	**Mermüller *et al.* (1977)**	**Anderberg (1991)**
Tribe Inuleae Sections: -Inuleae-Gnaphalieae * -Inulae-Archetypae* -Inuleae-Buphthalmeae	Tribe Inuleae Subtribes: -Filagineae* -Gnaphalieae* -Angianthieae* -Relhanieae* -Athrixieae*	Tribe Inuleae Subtribes: -Inulinae -Gnaphaliinae* -Athrixiinae*	Tribes: -Inuleae -Plucheae -Gnaphalieae Gnaphalieae subtribes: -Angianthinae -Cassiniinae -Gnaphaliinae -Loricariinae -Relhaniinae

2. Phylogenetic relationships within the Gnaphalieae. The "*Relhania* clade"

As reported by Ward *et al.* [6], there are three main clades within the Gnaphalieae: (1) the "*Relhania* clade", which is sister to the rest of the Gnaphalieae s. str., as previously noted by Bayer *et al.* [14]; (2) the "*Dolichothrix-Phaenocoma* clade", which is the next one to diverge; and (3) the "crown radiation clade", which comprises the rest of the genera included,

up to now, in the Gnaphalieae phylogenies. According to Bayer *et al.* [14, 15] the fives subtribes recognized by Anderberg [4] are not monophyletic, implying that a recircumscription was necessary. The tribal and infratribal position of some genera of the Gnaphalieae, mainly those that belong to Anderberg's [4] "basal group", are another taxonomic problem. The "basal group" comprises an assemblage of genera previously included in the Inuleae-Athrixiinae or Inuleae-Gnaphaliinae by Merxmüller *et al.* [9], together with other genera considered plesiomorphic relatives of the Gnaphalieae [10]. In fact, according to the latest phylogenetic studies on the Gnaphalieae, some of those genera are nested in the "*Relhania* clade" [6]. The members of the "*Relhania* clade" and most of the genera of the "basal group" are mainly distributed throughout Southern and tropical Africa and Australia, with several representatives in Macaronesia and the Mediterranean region, North Africa, Arabia, Middle East and the Irano-Turanian region [4], see Fig. 1. The "crown radiation clade" included genera previously assigned to the different subtribes delimited by Anderberg [4] and some of the genera included in the "basal group". However, the infratribal affinities of some of the members of the "basal group" are unknown from a molecular point of view.

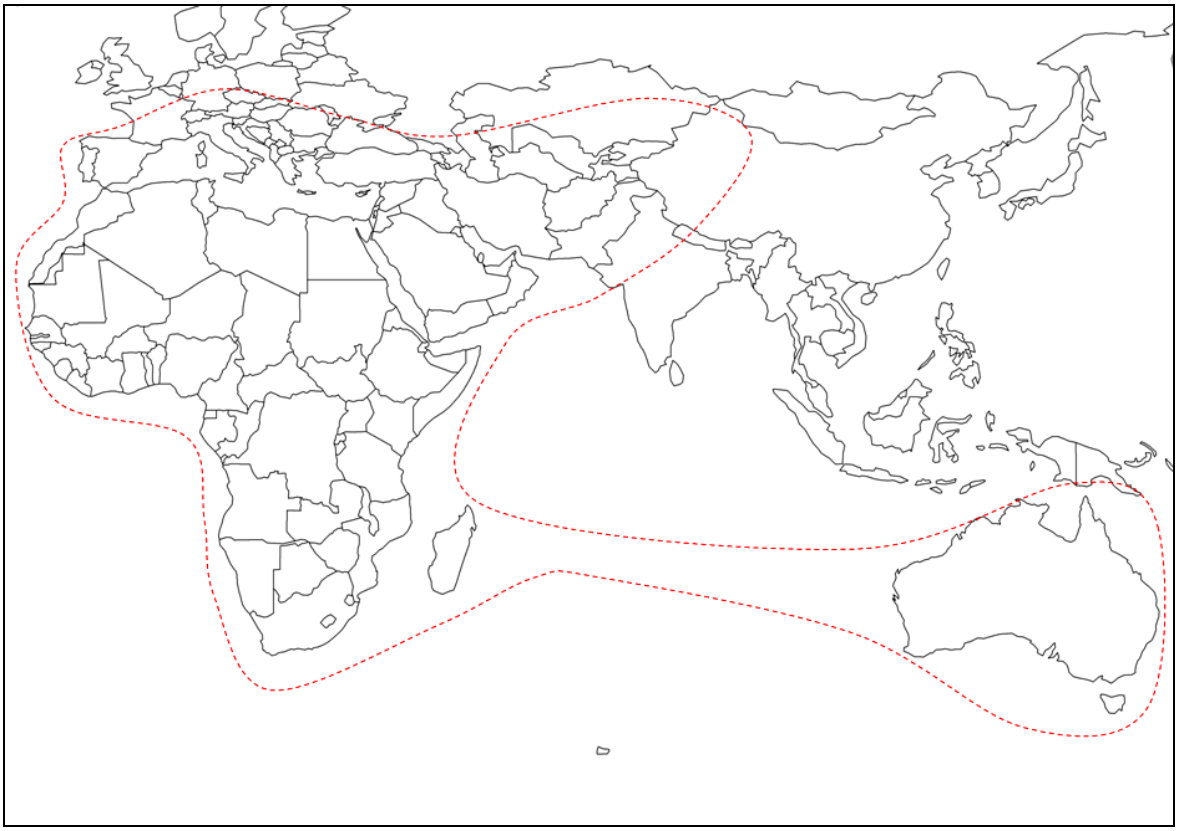

Figure 1. Distribution area of "*Relhania* clade" genera.

3. The genera *Aliella* and *Phagnalon*

The genus *Phagnalon* is distributed throughout Northeastern tropical Africa, the Macaronesian region, the Mediterranean basin, the Irano-Turanian region and the Saharo-Arabian region, but its greatest diversity is found in the Arabian Peninsula; it comprises about 36 species [16]. *Phagnalon* species are suffruticose shrubs or subshrubs and grow mainly in rocky areas, in a wide range of habitats: from sea level to 4.140 m altitude (Fig. 2). Species are morphologically characterized as having heterogamous disciform capitula;

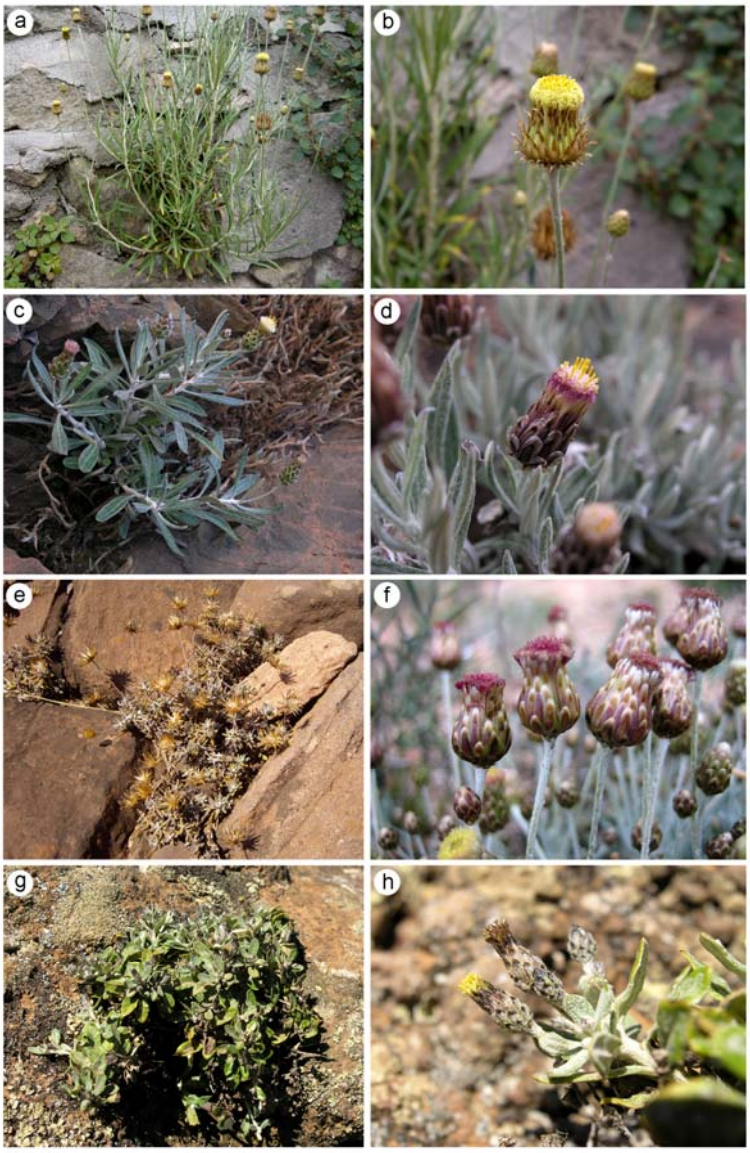

Figure 2. Habit and morphology of capitula in *Phagnalon* taxa. a and b) *Ph. saxatile* (L.) Cass. (N. Montes-Moreno); c and d) *Ph. bicolor* Ball (M.Galbany-Casals); e) *Ph. rupestre* (L.) DC. (N. Montes-Moreno) and f) *Ph. rupestre* (L.) DC. (L. Sáez); g and h) *Ph. melanoleucum* Webb (M. Galbany-Casals).

female florets outnumbering the hermaphrodite ones; involucral bracts with undivided stereome; receptacle flat; anthers provided with long or medium tails; style bifid with obtuse or acute sweeping hairs; achenes with duplex hairs and monomorphic and uniseriate pappus composed by barbellate bristles. As regards to chromosome number, all the cytologically known species of *Phagnalon* are diploids with $2n = 18$.

Cassini [17a,b] separated *Phagnalon* from *Gnaphalium* based on the morphology of the involucral bracts and the ecaudate anthers. The only infrageneric classification of *Phagnalon* was proposed by Maire [18] who recognized two sections, *Phagnalon* sect. *Gnaphaliopsis* and sect. *Euphagnalon* (Table 1). Section *Euphagnalon* was characterized by ecaudate anthers and comprised *Ph. bicolor* Ball, *Ph. calycinum* (Brouss. ex Cav.) DC., *Ph. iminouakense* Emb., *Ph. latifolium* Maire, *Ph. rupestre* (L.) DC., *Ph. saxatile* (L.) Cass., and *Ph. sordidum* (L.) Rchb. *Gnaphaliopsis* section was characterized by caudate anthers and encompassed *Ph. embergeri* Humbert & Maire, *Ph. helichrysoides* Coss. & Maire and *Ph. platyphyllum* Maire. Subsequently, these three species were transferred to the genus *Aliella* by Qaiser & Lack [19]. *Phagnalon iminouakense* was combined under *Aliella* by Dobignard [20].

The genus *Aliella* (Compositae, Gnaphalieae) was described as a segregate from *Phagnalon* [19]. It is currently accepted as an independent genus [4, 20, 5, 6]. *Aliella* comprises four species and two subspecies: *A. ballii* (Klatt) Greuter [≡ *A. helichrysoides* (Ball) Qaiser & Lack]; *A. ballii* subsp. *ballii*; *A. ballii* subsp. *nitida* (Emb.) Qaiser & Lack; *A. embergeri* (Humbert & Maire) Qaiser & Lack; *A. iminouakensis* (Emb.) Dobignard & Jeanm. and *A. platyphylla* (Maire) Qaiser & Lack (Table 2).

Table 2a. Major taxonomic treatments of species included in *Phagnalon* sect. *Gnaphaliopsis* and *Aliella.*

Maire (1928)	**Emberger (1932, 1935)**	**(Quézel, 1951)**
Ph. helichrysoides (Ball) Coss. ex Maire	*Ph. helichrysoides*	*Ph. helichrysoides*
	Ph. helichrysoides var. *nitidum* Emb.	–
Ph. platyphyllum (Maire) Maire	*Ph. platyphyllum*	*Ph. platyphyllum*
Ph. embergeri Humbert & Maire	*Ph. embergeri*	*Ph. embergeri*
	Ph. lepineyi Emb.	–
		Ph. iminouakense Emb.

Table 2b. Major taxonomic treatments of species included in *Phagnalon* sect. *Gnaphaliopsis* and *Aliella* (cont.).

Qaiser & Lack (1986a)	**Anderberg (1991)**	**Dobignard (1997)**	**Greuter (2008)**
A. helichrysoides (Ball) Qaiser & Lack	*A. bracteata* Anderb.	*A. helichrysoides*	*A. ballii* (Klatt) Greuter
A. helichrysoides Qaiser & Lack subsp. *nitida* (Emb.) Qaiser & Lack	–	*A. helichrysoides* subsp. *nitida*	*A. ballii* subsp. *nitida* (Emb.) Greuter
A. platyphylla (Maire) Qaiser & Lack	*A. platyphylla*	*A. platyphylla*	*A. platyphylla*
A. embergeri (Humbert & Maire) Qaiser & Lack	*A. embergeri*	*A. embergeri*	–
–	–	–	–
–	–	*A. iminouakensis* (Emb.) Dobignard & Jeanm.	*A. iminouakensis*

The generic diagnosis was based on the presence of bracts on the peduncle similar in shape and size to the involucral bracts, the presence of waxy cushions on the corolla lobes, tubular female florets, caudate anthers, and pappus bristles barbellate from the base to the apex. The species of *Aliella* are caespitose or decumbent chasmophytic endemics and grow in calcareous or siliceous rock crevices in the Atlas Mountains of Morocco, at altitudes from 1.800 to 3.630 m (Fig. 3).

Regarding chromosome number, all the reports for *Aliella* show $2n = 18$ [21, 22, 23], although there is a conflicting report of $2n = 14$ for *A. ballii* [22].

According to the results of an analysis of morphological characters, the affinities of *Aliella* and *Phagnalon* are unknown [10]. *Phagnalon* was first placed in the tribe Inuleae, subtribe Gnaphaliinae, series Eugnaphalieae [8]. Later, it was accommodated in an informal group in the treatment of Inuleae by Merxmüller *et al.* [9], because it has some intermediate characters between the Gnaphaliinae, Inuliinae and Athriixinae; these features include filiform female florets, cartilaginous involucral bracts, apically confluent stigmatic areas, tailless anthers, a particular geographic distribution and a high basic chromosome number. Anderberg [10] placed *Aliella* and *Phagnalon*

Figure 3. Habit of *Aliella* taxa: a) *Aliella platyphylla* (Maire) Qaiser & Lack (L. Sáez); b, c and d) *Aliella ballii* subsp. *ballii* (N. Montes-Moreno); e) *Aliella ballii* subsp. *nitida* (Emb.) Greuter (N. Montes-Moreno); f) *Aliella iminouakensis* (Emb.) Dobignard & Jeanm. (N. Montes-Moreno).

in the Gnaphalieae based on morphological data. However, the results did not clearly indicate the subtribal affinities of *Aliella* and *Phagnalon* but pointed out that *Phagnalon* was nested within a clade with *Anisothrix* O. Hoffm., whereas *Aliella* was more related to the majority of the taxa of the Gnaphalieae than to *Phagnalon*. Therefore, Anderberg [4] included *Aliella* and *Phagnalon* in "the basal group" of the Gnaphalieae, together with the genera *Anisothrix*, *Pentatrichia* Klatt and *Philyrophyllum* O. Hoffm., among others. Up to date, tribal affinities of *Aliella* and *Phagnalon* have not been investigated with molecular markers.

4. Applications in the field of pharmacology and medicine

Some *Phagnalon* species have economic importance as they are a source of compounds with several applications in the field of pharmacology and medicine. According to Góngora [24a] and Góngora *et al.* [24b], different compounds were isolated from *Ph. rupestre* (L.) Cass. Some of them, mainly flavonoids and hydroquinone glucosides, produced reduction of inflammatory reactions. Ali-Shtayeh *et al.* [25] investigated the antimicrobial activities of 20 Palestinian plants against five bacterial species (*Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Pseudomonas aeruginosa*) and one yeast (*Candida albicans*). The results showed that the most antimicrobial active plants were *Ph. rupestre* and *Micromeria nervosa* (Desf.) Benth. Other phytochemical studies have isolated phenolic compounds with antioxidant activity from *Ph. rupestre* [26]. Similarly, other compounds were isolated from other *Phagnalon* species. For example, thymol derivatives were isolated from *Ph. sinaicum* Bornm. & Kneuck. [27] and l-chiro-inositol has been isolated from *Ph. sordidum* (L.) Rchb. [28]. According to Nuissier *et al.* [29] the chiro-inositol can be used in managing the diabetes. Other phenolic compounds were isolated from *Ph. saxatile* (L.) Cass. with applications in the treatment of Alzheimer's disease according to Conforti *et al.* [30]. In the Balearic Islands, *Ph. sordidum* is used alone or mixed with *Lippia citrodora* (Gómez Ortega & Palau) Kunth or *Malva sylvestris* L. to treat renal calculosis [28].

5. Material and methods of the phylogenetic study

Given the lack of phylogenetic studies on *Aliella* and *Phagnalon* in the literature, Montes-Moreno *et al.* [31] performed a combined nuclear and chloroplast phylogeny to check the hypothesized monophyly of *Aliella* and *Phagnalon* to provide more information on the position of both genera within the tribe Gnaphalieae, and on their interspecific relationships. This approach was chosen because non-homologous morphological similarities are frequent in the Gnaphalieae [14]. Moreover, combined nuclear and chloroplast phylogenies have been successfully used in the Compositae and the Gnaphalieae at the generic and specific levels [14, 15, 32, 33, 34, 35, 36, 37].

Montes-Moreno *et al.* [31] sampled 29 of the 36 species of the genus *Phagnalon* together with four *Aliella* species. Representatives of the "*Dolichothrix-Phaenocoma* clade", "crown radiation clade", "*Relhania* clade" and the "basal group", with emphasis on those that were already known to be placed within the "*Relhania* clade", were also included. Outgroups were chosen from the Heliantheae, Inuleae, Calenduleae,

Anthemideae and Astereae for the analysis of the *trnL* intron and the *trnL/F* intergenic spacer.

The phylogenetic analyses were performed with the five following data sets: (1) *trnL* intron and *trnL/F* intergenic spacer regions (Fig. 4) to: (i) elucidate the tribal position of *Aliella*, *Macowania*, *Phagnalon* and *Philyrophyllum* and to (ii) determine the relationships between *Aliella* and *Phagnalon* and the early branching genera of the "*Relhania* clade"(2) ETS+ITS+ycf3-trnS+trnT-trnL (Fig. 5) to study the generic limits of *Aliella* and *Phagnalo*n, and to elucidate the relationships within the *Aliella* and *Phagnalon* taxa (3) ETS+ITS (Fig. 6), in order to examine the position of some taxa not included in previous analyses and for which we could not amplify any chloroplast region (4) ycf3-trnS+trnT-trnL (Fig. 7) to verify the relationships between *Aliella* and *Phagnalon* and to elucidate possible incongruities with the nrDNA data and (5) ETS+ITS, performed with the exclusion of *Ph. latifolium* and *A. iminouakensis* (Fig. 8).

To analyze the combined cpDNA data set (*ycf3-trnS+trnT-trnL*), nrDNA data set (ETS+ITS) and nr-cpDNA data set (ETS+ITS+*ycf3-trnS+trnT-trnL*), *Anisothrix*, *Pentatrichia* and *Athrixia* were selected as outgroups, because they were the closest genera to *Aliella* and *Phagnalon* according to the results obtained from the *trnL* intron and the *trnL/F* intergenic spacer analyses carried out (Fig. 4).

The analyses used 221 new sequences (40 ETS, 49 3'ETS, 40 ITS, 34 *ycf3-trnS*, 34 *trnT-trnL* and 24 *trnL* intron and the *trnL/F* intergenic spacer) together with 47 sequences from published EMBL/GenBank accessions [12, 14, 15, 38, 39, 40]; and also unpublished ones [Boelch *et al.* (unpublished) and Panero *et al.* (unpublished)]. Sources of published sequences, voucher data, and GenBank sequence accession numbers are given in Montes-Moreno *et al.* [31].

The protocols for DNA extraction, DNA amplification strategies, sequencing and phylogenethic analyses were described in Montes-Moreno *et al.* [31].

6. Phylogenetic relationships in the "*Relhania* clade"

Here we present the main relationships in the "*Relhania* clade" according to Montes-Moreno *et al.* [31].

The results from the combined *trnL* intron and *trnL-trnF* intergenic spacer pointed out that *Macowania* belongs to the "*Relhania* clade", given that *Macowania tenuifolia* forms a monophyletic group with *Arrowsmithia* (Fig. 4) within that clade. *Macowania* Oliv. consists of 12 species from South Africa, Arabia and Ethiopia [4], and it was already stated to be close to the "*Relhania* clade" on the basis of morphological data [4, 41, 42].

Our results strongly supported that *Philyrophyllum* O. Hoffm., a genus with two species endemic to Namibia and Botswana [43], belongs to the "crown radiation clade", in contrast with evidences from morphological characters which led Anderberg [4, 44] to consider it part of the "basal group", pointing out a close relationship with *Anisothrix* and *Pentatrichia*.

Athrixia Ker.-Gawl. comprises about 14 species from South Africa, tropical Africa and Madagascar [41]. Some authors pointed out that the phylogenetic affinities of this genus were outside of "*Relhania clade*" [14], while other authors argued that it was close to the "*Relhania* clade" [6].

Our results were in accordance with Ward *et al.* [6], because the four *Athrixia* species sampled formed a strongly supported monophyletic group within the *Relhania* clade (Fig. 4). These incongruent results between the different studies could be due to the different species sampled and indicate a possible problem of generic delimitation in *Athrixia*.

According to Montes-Moreno *et al.* [31], *Phagnalon* and *Aliella* are nested within the "*Relhania clade*", as they formed a monophyletic group together with *Anisothrix*, *Athrixia*, and *Pentatrichia.* An African origin for the ancestor of *Aliella* and *Phagnalon* was suggested because the genera more closely related to them are restricted to South Africa, tropical Africa and Madagascar, as it is the case of other Gnaphalieae with Mediterranean representatives (e.g., *Helichrysum* Mill., [40]; *Ifloga* Cass. and *Leysera* L., [39]).

Although the lack of informative characters did not allow to establish their sister group, the unambiguous alignment of 5'ETS sequences of *Aliella*, *Anisothrix, Pentatrichia* and *Phagnalon* suggested that *Anisothrix* and *Pentatrichia* are the most closely related to *Aliella* and *Phagnalon*, while the rest of representatives from the "*Relhania* clade" are more distantly related. This hypothetically sister relationship agreed with morphological and phytochemical data, as *Anisothrix*, *Pentatrichia* share with at least some *Aliella* and *Phagnalon* several morphological features: long caudate anthers, waxy cushions on the outside of the corolla lobes, non myxogenic filiform twin hairs on the achene surface, acute sweeping hairs arranged apically on the stigmatic surface (Montes-Moreno, pers. obs.), leaves with dentate margins [14], phloem not concealed in fibers as well, and presence of six derivatives of leysseral compounds among *Anisothrix* and *Phagnalon* [10,45].

7. Generic delineation of *Aliella* and *Phagnalon*

Montes-Moreno *et al.* [31] found that *Phagnalon* and *Aliella* form a strongly supported monophyletic group (Figs. 4–8), but the generic boundaries between them were difficult to establish because the results showed unresolved phylogenetic relationships. Firstly, only three *Aliella*

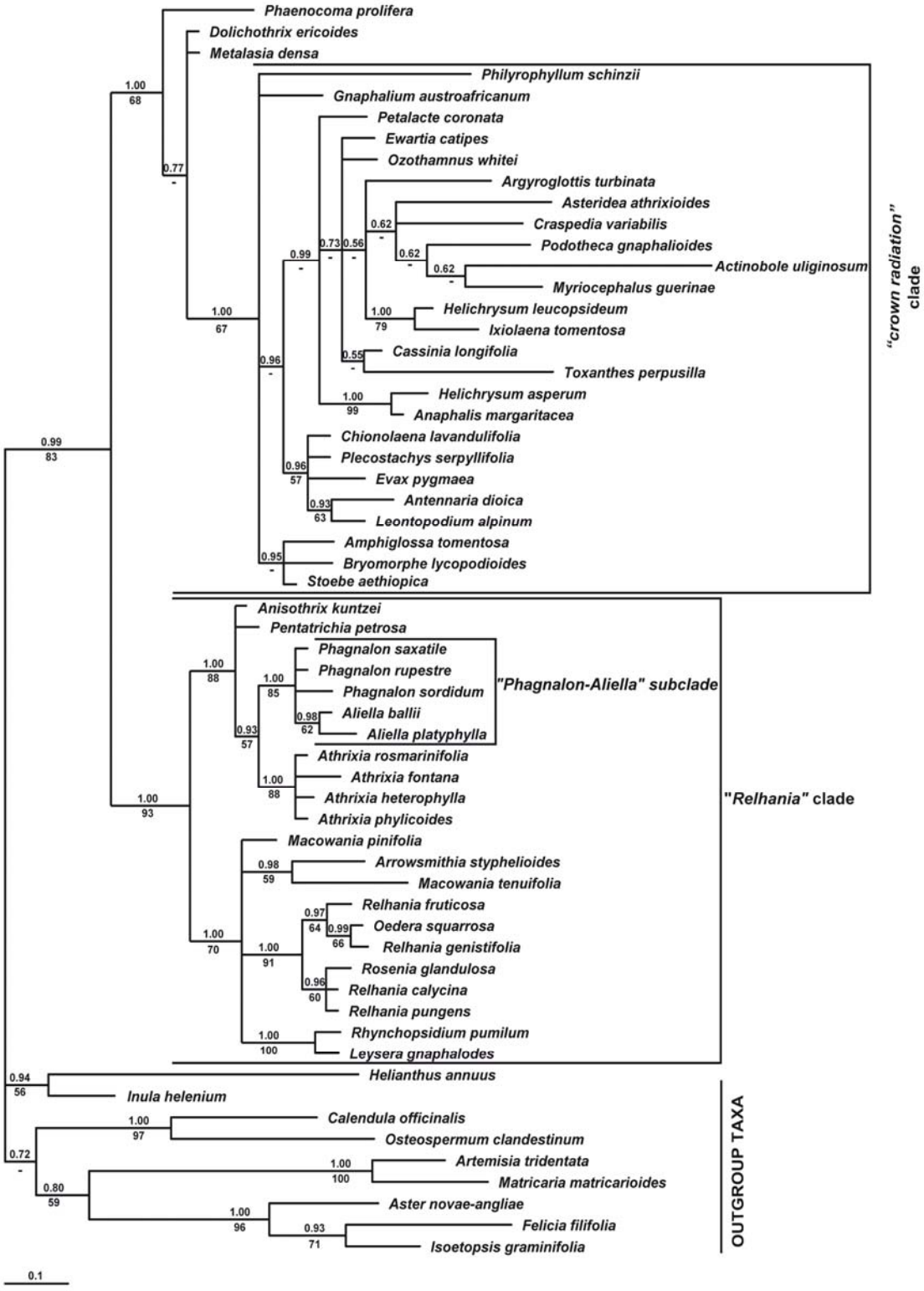

Figure 4. Strict consensus tree of the 1.516 equally parsimonious trees obtained in the heuristic search from the *trnL* intron and *trnL-trnF* intergenic spacer sequence data. Numbers below the branches indicate parsimony bootstrap percentages (BS). Numbers above the branches are Bayesian clade-credibility values (posterior probability, PP).

species (*A. ballii*, *A. embergeri* and *A. platyphylla*) formed a monophyletic group; (Figs. 5 and 6). Secondly, there were incongruities between the molecular and morphological data because *A. iminouakensis* was firmly nested within the *Phagnalon* clade, even though presented all the diagnostic characters of *Aliella* (Fig. 7). Finally, the monophyly of *Phagnalon* was only statistically supported by the analysis carried out excluding *A. iminouakensis* and *Ph. latifolium* (Fig. 8).

These results suggested that *A. iminouakensis* and *Ph. latifolium* have a complex phylogenetic history and could be derived from an ancient hybridization event for the following reasons: a) the noteworthy increase in the bootstrap value of the *Phagnalon* clade, after the exclusion of both taxa; b) the incongruities between nrDNA and cpDNA data sets indicated by the ILD test and the tree topologies; c) the unresolved position of these two taxa; and d) the morphological intermediacy of *Ph. latifolium* between *Aliella* and *Phagnalon.*

Hybridization has been documented among several *Phagnalon* species (*Ph. saxatile*, *Ph. sordidum* and *Ph. rupestre*, cf. [46]; and *Ph. niveum* Edgew. and *Ph. pycnophyllon* Rech. f., cf. [16]; Montes-Moreno, pers. obs.), from the observation of several intermediate specimens in the field and in herbarium material. The geographical distribution of *A. iminouakensis* does not currently overlap with the putative *Phagnalon* maternal (cpDNA) donors (Fig. 6), but the geographical distribution of *Ph. latifolium* overlaps with *A. ballii* and *A. platyphylla*, since vouchers of *A. ballii*, *A. platyphylla* and *Ph. latifolium* have been collected in the same locality. Moreover, other hybridization or introgression events have also been reported between taxa included in different sections or genera in the Compositae (*Andryala* L., *Hieracium* L., and *Pilosella* Hill., cf. [47]), and particularly in the Gnaphalieae [*Anaphalioides* (Benth.) Kirp. and *Ewartia* Beauverd, cf. [48]; *Anaphalioides* and *Helichrysum*, cf. [49]].

Further studies involving genetic markers are required to confirm the putative hybridization hypothesized for *Phagnalon* and *Aliella*.

8. Phylogenetic relationships in *Phagnalon*

Results of the combined nuclear-chloroplast phylogeny indicated that the diversification in *Phagnalon* took place in three main areas [31]: the Irano-Turanian region, the Mediterranean and Macaronesian regions, and the Saharo-Arabian region. The acronyms used in all the phylogenetic trees are: AS Asian, AAT AntiAtlas, AP Arabian Peninsula, C central, CI Canary Islands, CR Crete, CV Cape Verde, E Eastern, ET Ethiopia, HAT High Atlas, IR Iran, IT Irano-Turanian, MAC Macaronesian, MAT Middle Atlas, MED Mediterranean, NA North African, SA Saharo-Arabian, SI Sinai, W western, YE Yemen.

The Irano-Turanian Clade included species from the Irano-Turanian region and *Ph. pygmaeum* (Sieber) Greuter, a Crete endemic which is sister to the rest of the Irano-Turanian species (Figs. 5, 6 and 8). This relationship suggested that a common ancestor of the whole Irano-Turanian clade reached Crete and, from there, colonized the mainland, where it radiated and diversified in the Irano-Turanian region by wind dispersal of the achenes, as Qaiser & Lack (1986a) reported for other *Phagnalon* species.

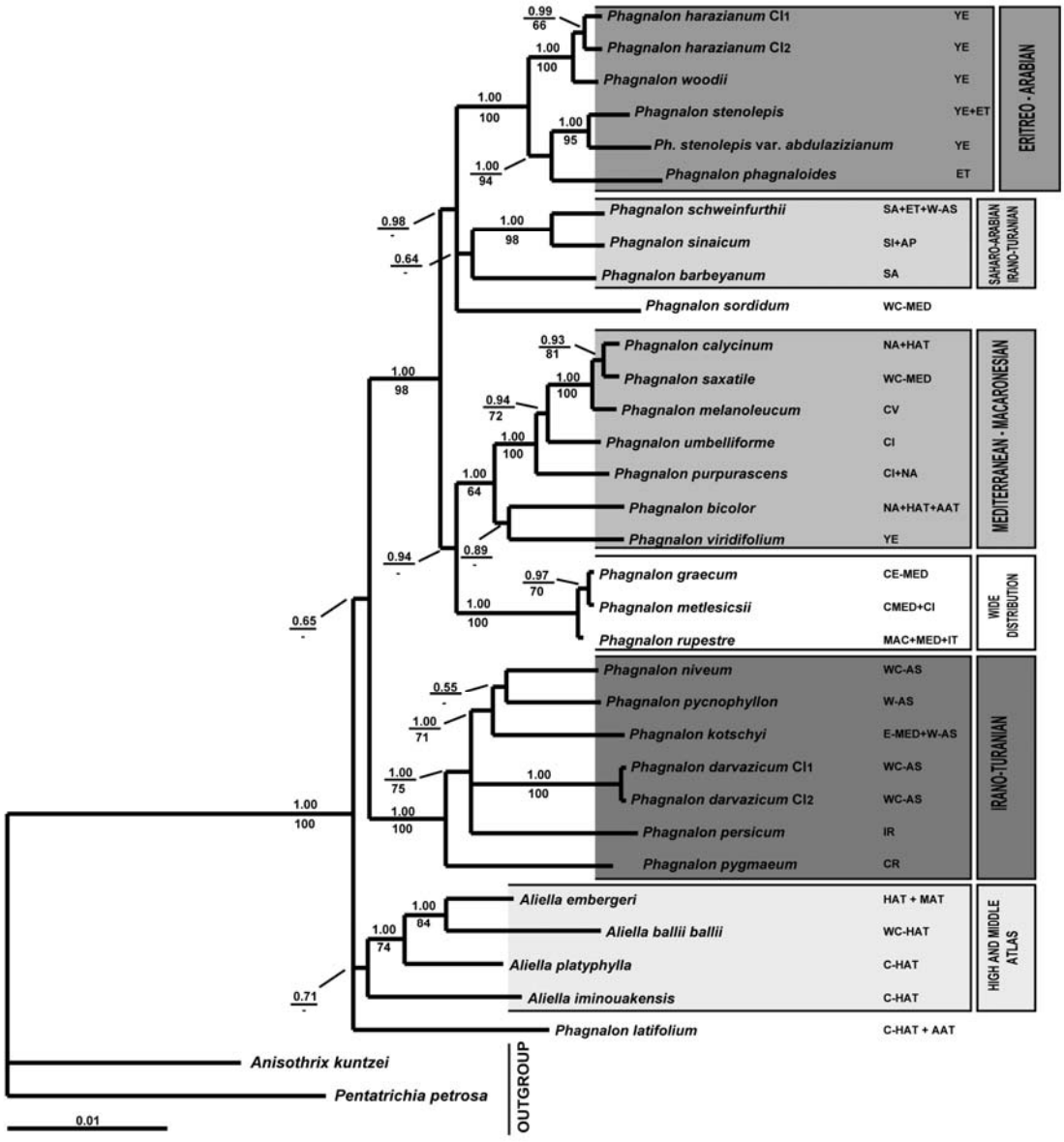

Figure 5. Consensus phylogram of the post-burn-in trees resulting from Bayesian MCMC analysis of the combined ETS, ITS, *ycf3-trnS* and *trnT-trnL* data sets. Numbers above the branches are Bayesian clade credibilities (posterior probability, PP). Numbers below the branches indicate parsimony bootstrap percentages (BS). Other abbreviations: Cl = clone; Pop = population.

However, a second possible scenario may have involved a common ancestor, distributed and diversified throughout the mainland that colonized Crete, via land connections or long distance dispersal, before becoming extinct. In addition, a combination of both scenarios was also possible.

The interspecific relationships among these Irano-Turanian taxa could be explained by considering some morphological affinities: *Ph. acuminatum* Boiss., *Ph. kotschyi* Sch. Bip. ex Boiss. and *Ph. pygmaeum* have linear to subulate bracts in the capitulum; *Phagnalon kotschyi*, *Ph. pygmaeum* and *Ph. persicum* Boiss. have waxy cushions on the outside of the corolla lobes. The lack of resolution of the Irano-Turanian clade was mainly attributed to recent diversification or to the putative hybridization between *Ph. niveum* and *Ph. pycnophyllon* [16].

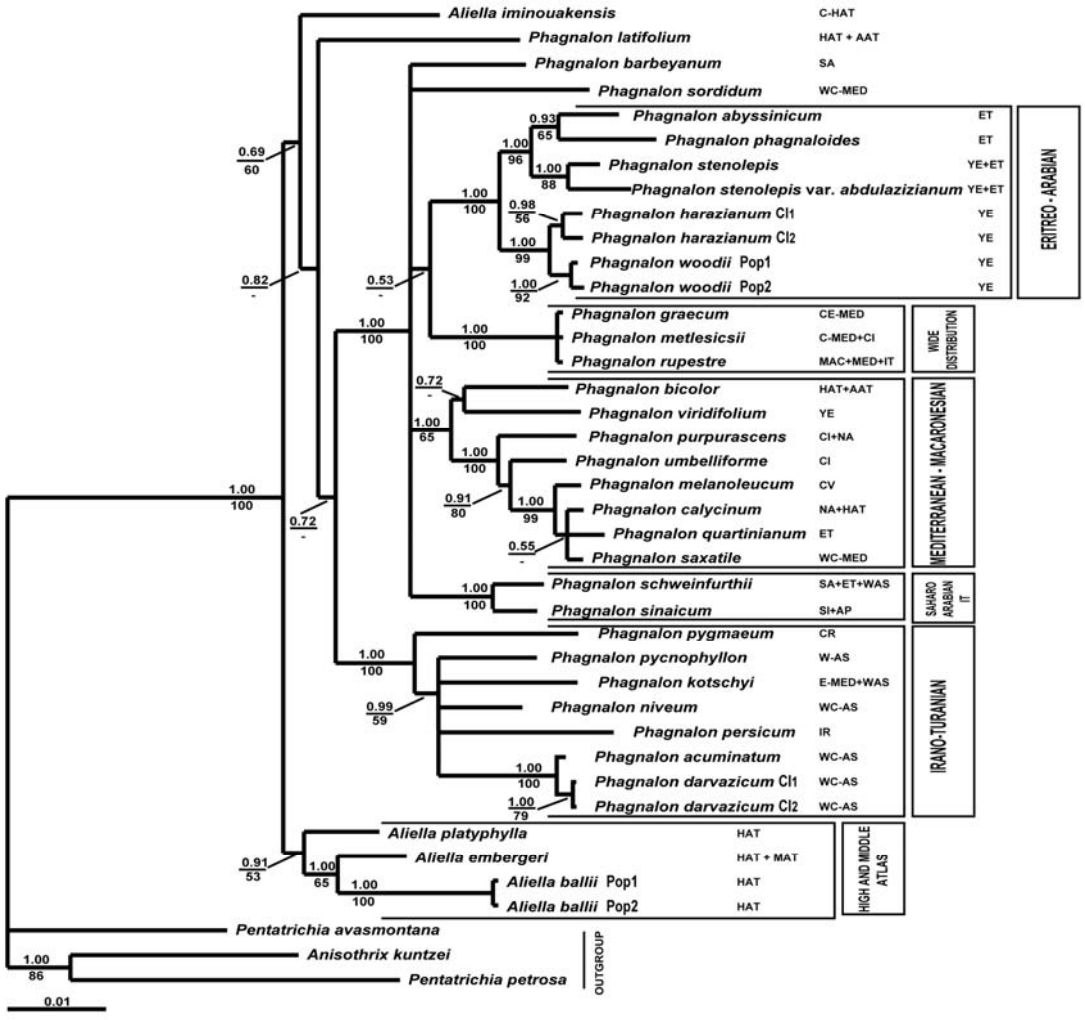

Figure 6. Consensus phylogram of the post-burn-in trees resulting from Bayesian MCMC analysis of the combined ETS and ITS data sets. Numbers above the branches are Bayesian clade credibilities (posterior probability, PP). Numbers below the branches indicate parsimony bootstrap percentages (BS). Other abbreviations: Cl = clone; Pop = population.

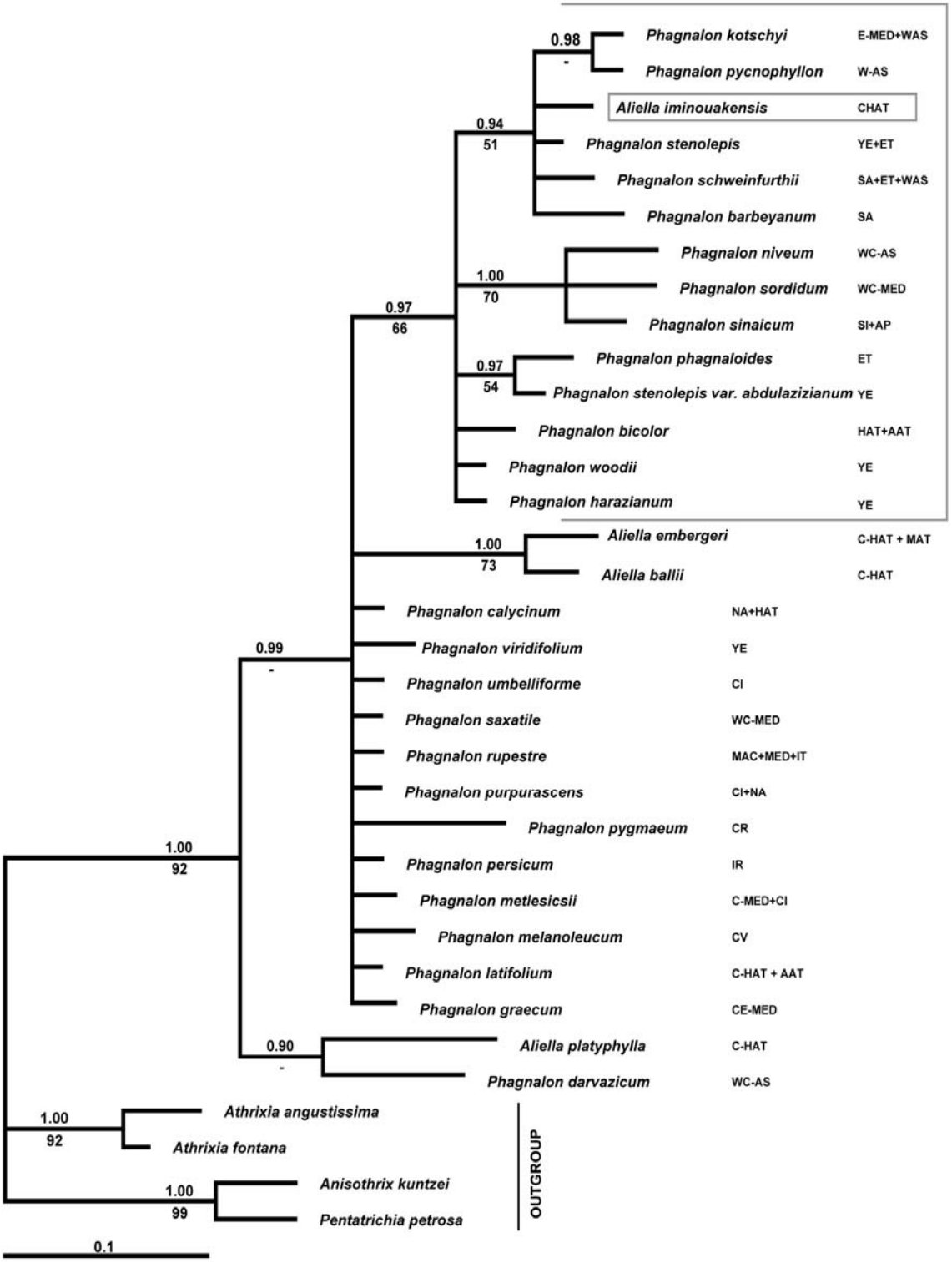

Figure 7. Consensus phylogram of the post-burn-in trees resulting from Bayesian MCMC analysis of the combined chloroplast *ycf3-trnS* and *trnT-trnL* sequence data. Numbers above the branches are Bayesian clade credibilities (posterior probability, PP). Numbers below the branches indicate parsimony bootstrap percentages (BS). Other abbreviations: Cl = clone; Pop = population.

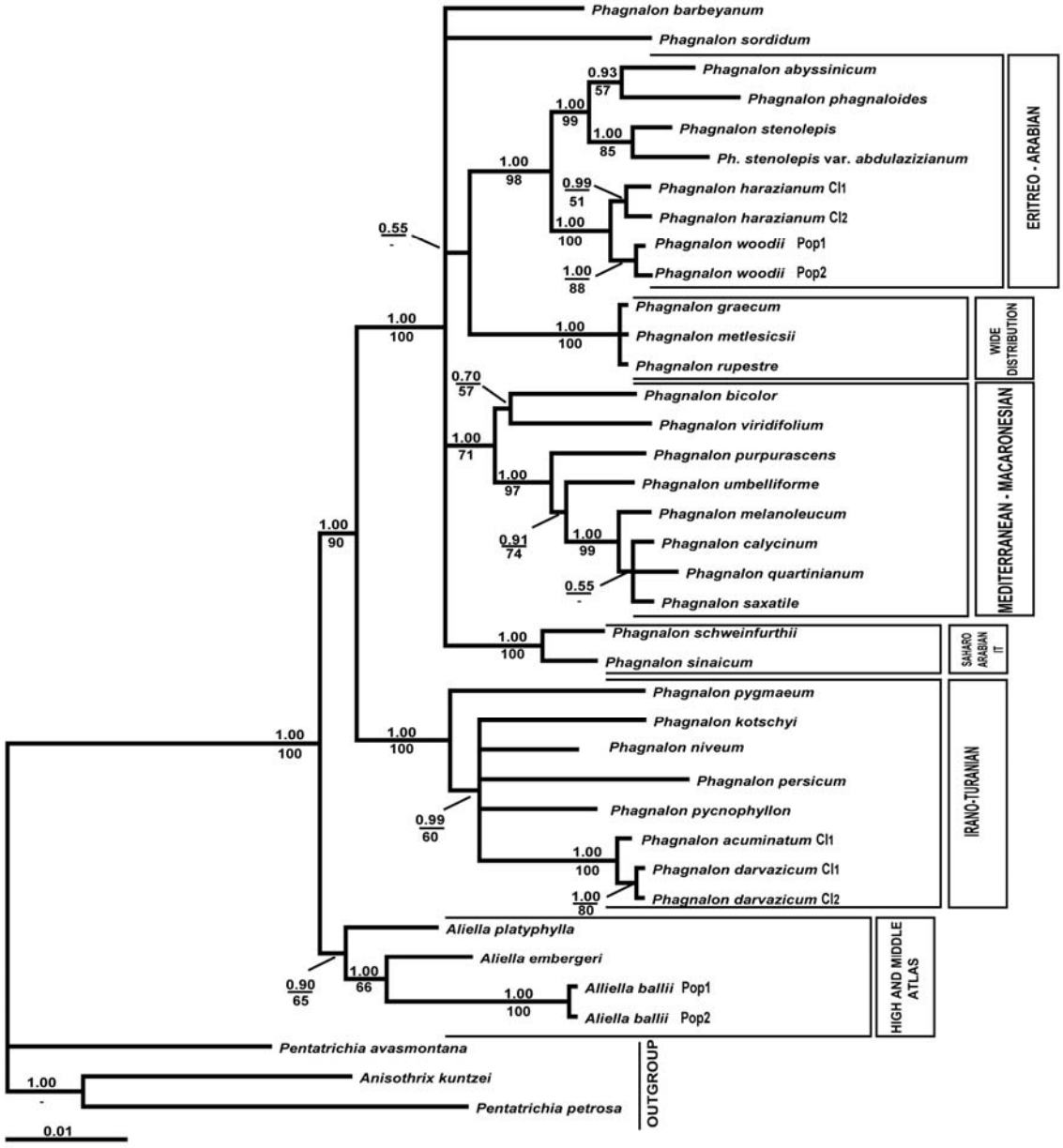

Figure 8. Consensus phylogram of the post-burn-in trees resulting from Bayesian MCMC analysis of the combined ETS and ITS sequence data performed with the exclusion of *Aliella iminouakensis* and *Phagnalon latifolium*. Numbers above the branches are Bayesian clade credibilities (posterior probability, PP). Numbers below the branches indicate parsimony bootstrap percentages (BS). Other abbreviations: Cl = clone; Pop = population.

The Mediterranean and Macaronesian Clade encompassed mostly Mediterranean and Macaronesian taxa. This clade included some East African and Saudi Arabian-Omani endemics (*Ph. quartinianum* A. Rich. and *Ph. viridifolium* Decne. ex Boiss. respectively; Fig. 6 and 8) and a subclade involving *Ph. purpurascens* Sch. Bip., *Ph. umbelliforme* DC., *Ph. melanoleucum* Webb, *Ph. calycinum* (Brouss. ex Cav.) DC. and *Ph. saxatile* (Figs. 5, 6 and 8).

This pattern is found in phylogenetic studies of other genera: *Aeonium* Webb & Berthel., *Pulicaria* Gaertn. [50], *Euphorbia* L. [51], and *Helichrysum* [45]. Traditionally, these disjunctions have been considered evidences of a continuous flora that existed in the late Miocene [52, 53]. This flora was widespread across the Sahara, North Africa and Mediterranean regions and disappeared in the major climatic changes of the late Tertiary and Quaternary [52, 54, 55]. Some morphological affinities between several representatives of this clade were also reported: *Ph. bicolor* Ball, *Ph. quartinianum* and *Ph. viridifolium* share the transparent scarious margins of the bracts.

The subclade involving *Ph. purpurascens* (North Africa and Canary Islands), *Ph. umbelliforme* (Canary Islands), *Ph. melanoleucum* (Cape Verde), *Ph. calycinum* (North Africa) and *Ph. saxatile* (Western Mediterranean and Macaronesian region) suggested the two following hypotheses: (1) The Canary Islands were colonized by a North African ancestor by a long-distance dispersal event from North Africa, as has been hypothesized for other plant groups [45, 56]. A Canary Islands ancestor would have colonized the Cape Verde Islands, followed by a back colonization to the mainland, where *Ph. calycinum* and *Ph. saxatile* would have originated (Fig. 5, 6 and 8); (2) The second scenario involved an extinct ancestor distributed in North Africa that radiated to the Canary Islands, Cape Verde Islands and the Mediterranean area. The colonization of the Cape Verde Islands and Mediterranean region by North African floristic elements has been postulated by other authors [45, 57, 58]. In addition, the species distributed in the Canary Islands do not form a monophyletic group. Therefore, the Canary Islands colonization by *Phagnalon* would have been produced by, at least, two colonization events, as other phylogenetic studies have pointed out for some Macaronesian plant groups [45, 59].

The Eritreo-Arabian Clade included a second subclade encompassed mainly endemics to Yemen, and included *Ph. harazianum* Deflers and *Ph. woodii* Qaiser & Lack (Figs. 5, 6 and 8). This Eritreo-Arabian Clade also included *Ph. abyssinicum* Sch. Bip. ex A. Rich. and *Ph. phagnaloides* (Sch. Bip. ex A. Rich.) Cufod. (both endemic from Ethiopia) and *Ph. stenolepis* Chiov., which has a disjoint distribution between Sudan (Jebel Marra), Chad (Tibesti), Ethiopia, Saudi Arabia and Yemen (Fig. 5, 6 and 8). This result may suggest that a common ancestor of these three taxa reached either Yemen or Ethiopia and colonized these areas, probably by long-distance dispersal of the light achenes, as previously indicated by Wickens [60]. This dispersal event likely followed a West-to-East route, which is in accordance to the African migration routes hypothesized for the Pliocene and Pleistocene floras [52]. From a systematic point of view, our results are the first in confirming that *Ph. phagnaloides* belongs to *Phagnalon*, as this species is

very different from the rest of *Phagnalon* species in having the capitulum arranged in leafy racemes, and was considered by other authors [A. Richard and Schultz Bipontinus] under the genus *Blumea* and *Pluchea* respectively.

Another endemic from adjacent Saudi Arabian-Oman area (*Ph. quartinianum*) was clustered in the Mediterranean-Macaronesian clade together with East African *Ph. viridifolium*. This phylogenetic relationship suggested that the East African and Yemen-Oman areas underwent, at least, two colonization events, because all the remaining endemics to Arabia and Ethiopia are nested within the Eritreo-Arabian subclade.

Other highly supported monophyletic groups were not nested within the three main clades. One of them included *Ph. graecum* Boiss. & Heldr., *Ph. metlesicsii* Pignatti, and *Ph. rupestre* (Figs. 5, 6 and 8). *Phagnalon metlesicsii* was described from Sicily based on glabrous leaves with dentate margins, and some other glabrous populations of *Ph. rupestre* have been located in the Canary Islands [61]. Based on the molecular, morphological and chorological data, *Ph. metlesicsii* was concluded to fall into the morphological variability of *Ph. rupestre*.

However, there were some incongruities between the morphological and molecular data: *Ph. stenolepis* and *Ph. acuminatum* have linear to lanceolate bracts and lanceolate leaves with dentate margins but did not form a monophyletic group. Likewise, *Ph. quartinianum*, *Ph. schweinfurthii* Sch. Bip. ex Schweinf., *Ph. sinaicum* Bornm. & Kneuck. and *Ph. viridifolium* have oblong to spathulate bracts, with broadly scarious transparent margins, but they did not form a monophyletic group. This result indicated that these morphological traits appeared more than once in *Phagnalon* and should be interpreted as morphological convergence.

9. Conclusion

The study of Montes-Moreno *et al*. [31] discussed in this review was the first to define, on a molecular basis, the phylogenetic position of *Aliella, Macowania* and *Phagnalon* in the "*Relhania* clade". In addition, the *Philyrophyllum* was found to be placed within the "crown radiation clade". Moreover, *Anisothrix*, *Athrixia* and *Pentatrichia* are the most closely related genera to *Aliella* and *Phagnalon*. The diversification in *Phagnalon* took place in three main areas: Western and Central Asia, the Mediterranean and Macaronesian region, and the Eritreo-Arabian region. Some incongruities between the chloroplast and nuclear molecular data, as well as the lack of resolution in some clades, suggested that hybridization could have played an important role in the evolution and diversification of both *Aliella* and *Phagnalon*.

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8. Occurrence of patulin in organic and conventional apple juice. Risk assessment

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Abstract. Organic foods are promoted as environmentally friendly and its consumption has become increasingly popular. Nevertheless, organic farming practices could produce commodities more susceptible to fungal attack and as a result, food with higher levels of mycotoxins. Patulin is a mycotoxin mainly present in apples and apple-based products. In this paper, we report our recent results and an update of the patulin occurrence and their risk in conventional and organic apple juices around Europe.

Introduction

Mycotoxins are secondary metabolites produced by some filamentous fungi. The main genera related to mycotoxins production are: *Alternaria, Claviceps, Fusarium, Aspergillus* and *Penicillium*. Some of them are responsible of the presence of mycotoxins in the crops (*Alternaria, Claviceps* and, *Fusarium*) and, some of them in post-harvest processes (*Aspergillus, Penicillium* and *Fusarium)* [1,2].

The term mycotoxin was used for the first time in 1962 after a veterinary crisis in England in which approximately 100,000 turkey poults died. A toxin

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produced by fungi was found responsible of this crisis. Up to now, there are around 300 different mycotoxins identified, but the most important for human health are aflatoxins, ochratoxins, tricotecenes, citrinin, patulin, zearalenone, ergot alkaloids and fumonisins [1].

In general, mycotoxicosis refers to toxic effects or poisoning due to mycotoxins. These effects can be acute and chronic effects and range from irritation to severe damage including hepatic and kidney toxicity, neurotoxicity, immunotoxicity, skin irritation, disorders of reproduction and endocrine systems, and carcinogenic effects. Nowadays, chronic toxicity of many mycotoxins, characterized by low-dose exposure over a long time period, is usually of greater concern than acute toxicity, particularly in developed countries [3].

The principal route of exposure to mycotoxins is through intake of contaminated food. Its presence in levels higher than tolerable represents a threat to food safety and a risk for human health. In fact, mycotoxins are the most important chronic food safety risk factor and of greater risk than synthetic food contaminants, plant toxins, food additives, or pesticide residues [4]. Although it would be impossible completely eliminate mycotoxins in food, it is important to ensure that their levels do not pose an excessive health threat. Consequently, regulations relating to mycotoxins in food and feed have been established. What is more, over the past years, several studies have been done in order to monitor the human exposure to mycotoxins and determine the associated risks [3].

Some of these monitoring studies include the analysis of organic food. This is not surprising considering that typical organic farming practices are characterized by very strict limits on chemical synthetic pesticide (fungicides, herbicides, insecticides) and synthetic fertilizer use. This fact could lead to produce food more susceptible to fungal attack and mycotoxin contamination. Must be taken into account that the most important factors that influence the presence of mycotoxins in food are the insect attack, damage to vegetables during harvest and the temperature and humidity during storage [3,5].

In the last years, consumption of organic foods have become increasingly popular for a number of reasons, the most significant of which is the perception of health and wellness benefits associated with naturally grown foods. Specifically, organic foods are promoted as being safer, better-tasting, environmentally and farmer friendly. In addition, consumers are choosing organic foods because several foods crisis have caused a health concern. Consequently, organic farming has been raised in Spain, especially in Catalonia, as well as in whole Europe, since the nineties. However, the health benefits of consuming organic compared to conventional foods are still

unclear. Current scientific literature cannot state that organic food is healthier than conventional alternatives and future research is needed [6].

In the following sections, the occurrence of patulin in organic and conventional apple juice in Europe and the risk of their intake are analyzed and the recent findings performed by our group are reported.

1. Patulin

Patulin is a mycotoxin produced by some filamentous fungi of the genus *Aspergillus, Penicillium* and *Byssochlamys*. These fungi can growth in fruits and vegetables, but the apples and apple-based products are considered the main source of this mycotoxin and *Penicillum expansum* the main responsible [7].

Chemically, patulin [4-hydroxy-4-H-furo(3,2-c)pyran-2(6H)-one], is a water soluble unsaturated heterocyclic lactone (Fig. 1) [8].

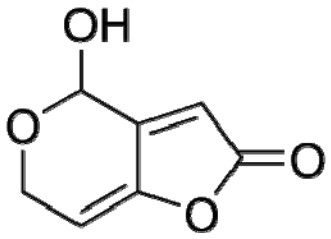

Figure 1. Chemical structure of patulin.

1.1. Toxic effects

Although patulin has antibiotic properties and it was firstly proposed for therapeutic purpose, in 1960s it was reclassified as mycotoxin due to its toxicity. Several toxicological studies have been done in order to characterize the patulin toxicity, only a few using *in vivo* models. In acute and short-term *in vivo* studies, patulin caused gastrointestinal effects as, distension, ulceration and haemorrhage. In chronic studies in rats, patulin caused neurotoxicity, immunotoxicity and genotoxicity. Reproductive and teratogenicity *in vivo* studies showed that patulin was embriotoxic. Regarding its potential as a human carcinogen, it has been classified as group 3 (Group 3- Not classifiable as to its carcinogenicity to humans) by International Agency for Research on Cancer (IARC) [8]. Recent studies have also demonstrated that patulin alters the intestinal barrier function [9].

Patulin has electrophilic properties, thus high reactivity to cellular nucleophiles. It is believed to exert its cytotoxic and chromosome-damaging effects mainly by forming covalent adducts with essential cellular thiols

especially with sulfhydryl groups. These properties, led to cause cross linking with proteins, enzyme inhibition, depletion of glutathione and genetic damage including chromosomal aberrations [10-12].

1.2. Regulatory aspects

Worldwide, regulations regarding to mycotoxins have been established to protect the consumer from the harmful effects of these compounds. Since 1981 the number of countries with specific mycotoxin regulations has been increased from 33 to 100 in 2003. In Europe, the first harmonized regulations for mycotoxins in human food came into force in 1998 [13].

Nowadays, patulin is one of the most regulated mycotoxins; many countries have regulation for this mycotoxin, especially in apple products like juice or puree. The great majority of these countries have established a 50 μg/kg as a reference level. In particular European legislation have been established a maximum permitted level of 50 μg/kg for patulin in apples juices, 25 μg/kg for apple puree and 10 μg/kg if these products are intended for infants (children under the age of 12 month) and young children (children aged between one and three years) (Commission Regulation 1881/2006) [14].

2. Occurrence of patulin in apple juice

The occurrence of patulin in apple-based products like apple juice is of health concern, thus, it is important to monitor and control final products to confirm that patulin levels are below the established limits according to the European regulation, and also, check the population exposure and the derived risk.

The occurrence of patulin in apple juices, commercially available on many European countries has been reported, including Belgium [15,16], Denmark [17], Germany [18], Greece [19], Italy [20-25], Portugal [26,27], Spain [28-37], Sweden [38], and The Netherlands[39], which are summarized in Table 1.

The extent and the degree of patulin contamination in apple products, reported in these studies, are affected by a variety of factors including origin and characteristics of samples and the method of analysis. With regard to the samples, the food category (i.e. fruit apple, apple fruit from concentrate, apple nectar and mixed juices), the cloudiness (i.e. clear and cloudy), the production method (i.e. conventional, organic and integrated), the place of production (i.e. local or imported), and the production-size (industrial or handicraft-made) are usually taken into account. Referring to the method of analysis, another crucial factor, the type of extraction, purification and determination used are of great importance for the results.

The incidence of patulin is of great variability as a consequence of different criteria considering positive samples. For instance, some authors consider samples with patulin concentrations higher than limit of detection as positives samples [15,28,35] while others consider as positives, samples with patulin concentrations higher than limit of quantification [21,30].

The comparison of the results obtained by different authors about the patulin content in apple juices is complex and difficult due to the different analytical conditions, the information provided in the publications, and the number of samples analysed. Considering the mean value, none of the values

Table 1. Occurrence of patulin in apple juices reported in several European studies.

Country, region (study period)	Sample characteristics	N	% P	Max µg/kg or µg/L	Mean µg/kg or µg/L	Reference
Belgium, Flanders	Clear	67	16.4	-	14.1	[15]
	Cloudy	110	10.0	-	30.3	
	Conventional	90	13.3	-	10.2	
	Organic	65	12.3	65.7	43.1	
	Handcrafted	22	9.1	-	10.5	
	Total[a]	177	12	-	22.2	
Belgium (2001)	Clear	25	84	38.8	7.8	[16]
	Cloudy	18	77.8	26.1	10.7	
	Conventional	39	82.1	-	9.2	
	Organic	4	75	-	6.5	
	Belgian	29	79	38.8	10.3	
	Imported (France. Germany. Switzerland)	14	86	10.6	6.4	
	Industrial	32	81.3	24.2	7.0	
	Handicraft-made	11	81.8	38.8	14.6	
	Total	43	81.4	38.8	9.0	
Denmark, Copenhagen (2006)	Total	20	70	**122.5**	35.9	[17]
Germany (1996 – 1997)	Commercial	10	-	26.0	-	[18]
	Home-made	2	-	23.9	-	
	Total	12				
Greece (October 2004 – June 2006)	Total	29	100	11.8	5.6	[19]

Table 1. Continued

Country, region (study period)	Sample characteristics	N	% P	Max µg/kg or µg/L	Mean µg/kg or µg/L	Reference
Italy (2008)	Local	20	-	30.0	-	[20]
	Imported	15	-	30.0	-	
	Total[b]	35	88.6	30.0	18.1	
Italy (April – November 2005)	Clear	28	50.0	47.9	10.8	[21]
	Cloudy	25	44.0	44.9	7.6	
	Conventional	32	59.4	44.9	8.9	
	Organic	21	28.6	47.9	9.9	
	Total[b]	53	47.2	47.9	9.3	
Italy	Conventional	14	64.0	4.5	2.5	[22]
	Organic	8	38.0	22.0	9.8	
	Integrated	4	75.0	1.8	1.2	
	Total	26	58.0	22.0	-	
Italy (November 2003 – February 2004)	Conventional[c]	33	48	**53.4**	3.1	[23]
	Organic	24	50	**69.3**	7.1	
	Total	57	49	**69.3**	-	
Italy, Naples	Conventional[d]	7	42.9	**56.4**	30.4	[24]
	Organic	7	42.9	33.2	21.6	
	Integrated	1	0	0.0	0	
	Total	15	40	-	-	
Italy	Conventional	21	-	3.03	1.01	[25]
	Organic	21		28.2	7.7	
	Conventional & cloudy	12	83.33	**1150**	-	
	Total	33	-	-	-	
Portugal, Lisbon (August 2007-March 2009)	Clear[g]	32	12.5	5.5	-	[26]
	Cloudy	36	66.7	42.0	-	
	Conventional[e]	49	-	42.0	-	
	Organic	19	-	9.2	-	
	Total	68	41	42	-	
Portugal, Porto	Conventional	9	77.8	12.6	-	[27]
	Organic[f]	3	100	8.9	-	
	Total	12	83.3	12.6	-	
Spain, Valencia (2008)	Total	28	7.14	6.0	4.2	[28]
Spain, Navarra-Pamplona (2008)	Total[g]	20	70	27.09	-	[29]
		20	70	29.61	8	

Table 1. Continued

Country, region (study period)	Sample characteristics	N	% P	Max µg/kg or µg/L	Mean µg/kg or µg/L	Reference
Spain, Catalonia (June – July 2008)	Clear & conventional	71	42.3	15.0	8.1	[30]
	Conventional[g]	12	25	9.2	7.5	
	Total	83	39.7	15.0	-	
Spain, Navarra	Conventional	95	67.4	**118**	-	[31]
	Organic	5	60	17.4	-	
	Total	100	67	**118**	19.4	
Spain	Total	28	7.1	6.0	-	[32]
Spain, Navarra	Total	20	100	**107**	41.3	[33]
Spain, Valencia	Total[h]	17	29.4	**50.9**	-	[34]
Spain, Madrid (2004 – 2005)	Local	13	15.4	7.5	1.3	[35]
	France (southeast)	8	0.0	-	-	
	Portugal (north)	4	0.0	-	-	
	Total[i]	25	8.0	7.5	-	
Spain, Madrid (April – December 1992)	Local	60	-	**-**	-	[36]
	Local	40	-	-	-	
	Total	100	82	**170**	13.8	
Spain, Catalonia	Total	8	75	**78.0**	-	[37]
Sweden (1996 (autumn) – 1998 (summer))	Total	39	12.8	< 50	-	[38]
The Netherlands	Conventional	5	0	-	-	[39]
	Organic	5	20	-	-	
	Total	10	20	-	-	

Notes: N, number of samples; P, positive samples; numbers shown in bold are out of law limit; - data not given in the study; [a]including apple juices for babies; [b]juices with 50 %, less or more than 50 % of fruit content; [c]enclosed is 1 sample of mixture with milk; [d]one of them was claimed GMO; [e]enclosed are 10 infant drink; [f]enclosed are two apple nectar; [g]samples intended for infants; [h]enclosed apple-containing beverages; [i]samples intended for young children.

reported by these studies exceed the patulin maximum permitted level of 50 μg/kg or 10 μg/kg (labelled and sold as intended for infants and young children) established by the EU regulation. Nevertheless, taking into account the maximum levels found in samples, several surveys carried out in Spain, Italy, Belgium and Denmark have reported levels of patulin over 50 μg/kg.

Comparisons of the mean patulin contamination level in clear and cloudy apple juices were reported in Belgium [15,16], Italy [21] and Portugal [26] with disagreeing results. Most of these surveys found that the incidence of patulin were higher in clear juices than in cloudy ones [15,16,26]. Only one study [21] found more positive samples in cloudy juices. However, regarding to mean and maximum values of patulin found in samples, almost all studies reported higher values in cloudy juices. The solid parts of cloudy juices are richer in proteins compared to the liquid phase, and probably patulin could interact with these proteins. In some cases, depending on the analysis method, this patulin bound to proteins could not be detected during analysis producing an underestimation on patulin content [40], which could explain the different results obtained.

Regarding the analysis of organic and conventional apple juices, some reports performed in Belgium [15,16], Italy [21-25], Portugal [26-27], and The Netherlands [39] showed significantly differences and contradictory results. Baert [15] did not found significant differences between the incidence of patulin in conventional (13%) and organic (12%) apple juices marketed in Belgium, however, the mean concentration of patulin in contaminated samples was significantly higher in organic (43.1 μg/L) than in conventional (10.2 μg/L) apple juices. On the contrary, Tangni [16] found higher mean concentration of patulin (9.2 μg/L) in contaminated conventional apple juices than in organic ones (6.5 μg/L). According to the surveys carried out in Italy, all except one [24] found that organic apple juices were much more contaminated than conventional. The two Portuguese surveys [26,27] have found higher levels of patulin in conventional apple juices than in organic ones. Finally, one Dutch survey [39] showed higher incidence in organic apple juices than in conventional ones.

A recent study has been done by our group in order to analyse the content of patulin in apple juice from organic and conventional production systems in Catalonia (Spain). None of the studies previously done in Catalonia have compared this production systems regarding patulin contamination. In fact, only one study has been done in Spain analysing organic apple juice [31], but the number of organic samples analysed were very few comparing with those conventional and also, mean value of patulin was not reported. In our study, twenty-four samples were analysed. The results showed a higher incidence of

Table 2. Patulin exposure for consuming apple-based juices from European countries.

Country, region	Population group	Body weight (kg)	Consumption (mL/day or kg/day)	Apple juice characteristics	Patulin intake (µg/kg bw/day)	Percentage of the PMTDI[a]	Reference
Belgium. Flanders	Preschool children	-	200	Conventional	0.009	2.3	[41,42]
				Organic	0.019	4.8	
				Handcrafted	0.010	2.5	
Belgium	Children	10	200	All apple juices[b]	0.180	45.0	[16]
	Adults	60	-		0.030	7.5	
Greece	Children	20	0.1 – 0.25	Conventional[c]	0.020	5.0 – 12.5	[19]
	Adolescent	55	0.1 – 0.5		0.008-0.042	2.0 – 10.5	
	Adults	70	0.1 – 0.25		0.010-0.020	2.5 – 5.0	
Italy	Children	20	59.3	Conventional[d]	0.003	0.9	[23]
				Organic	0.014	3.5	
	Adolescents	52	40.7	Conventional[d]	0.001	0.2	
				Organic	0.004	0.9	
	Adults	70	16.4	Conventional[d]	0.000	0.1	
				Organic	0.001	0.3	
	Elderly	70	13.2	Conventional[d]	0.000	0.1	
				Organic	0.001	0.2	
Italy	Adults	60	500 – 1000	Organic	0.224[e]	60.0	[25]
	Adults	60	500 – 1000	Conventional & cloudy	9.6	2400.0	
Spain. Valencia	Infants	48.2	64	Conventional	0.002	0.5	[28]
	Adults	74.6	64	Conventional	0.03	0.8	

Table 2. Continued

Country, region	Population group	Body weight (kg)	Consumption (mL/day or kg/day)	Apple juice characteristics	Patulin intake (µg/kg bw/day)	Percentage of the PMTDI[a]	Reference
Spain. Navarra	Infants	10	130	Apple juices intended for infants	0.104	26.0	[29]
Spain. Catalonia	Babies	11.3	20.4	Conventional[f]	0.040	10.3	[30]
	Infants and teenagers	48.2	64.53		0.008	1.5	
	Adults	74.6	63.95		0.005	1.2	
Spain. Navarra	Babies	10	130	Conventional & organic	0.252	63.0	[31]
	Children	25	200		0.155	38.8	
	Adults	70	200		0.055	13.8	
Spain. Valencia	Children	20	50	Fruit juices[g]	0.012	3.0	[34]
	Adults	60	61		0.005	1.2	
	Babies	12	216		0.086	21.6	
	Adults	64	200		0.015	3.8	
Sweeden	Children	-	-	All juices[h]	0.009-0.024	2.25 – 6.0	[38]
	Adults	-	-		0.004-0.011	1.0 – 2.8	

Notes: -, data not given in the study; [a]Provisional Maximal Tolerable Daily Intake (PMTDI) 400 ng/kg bw; bw = body weight; [b]considering all apple juices (clear & cloudy; conventional & organic; local & imported; and industrial & handicrafted); [c]considering all fruit juices (apple, orange and mixed fruits); [d]considering all fruit juices (apple, pear, other) and fruit purees; [e]calculated with the maximum patulin concentration by Baretta; [f]considering all apple products (compote, multifruit compote and apple juices for babies); [g]enclosed apple-containing beverages; [h]considering all juices.

positives samples in organic juices (72.7%) when comparing with conventional (15.4%) ($p<0.05$, Chi Square Test). Moreover, the mean concentration of patulin was also higher in organic (9.3 μg/kg) than in conventional apple juices (1.4 μg/kg) ($p<0.01$, Mann Whitney Test). None of the samples were above of the EU maximum permitted level.

3. Risk assessment

Few studies, concerning risk assessment of daily patulin intake through the consumption of apple juices, have been carried out in different European countries, including Belgium [41,42], Greece [19], Italy [23,25], Spain [28-31,34] and Sweden [38]. The key data of all studies are shown in Table 2.

The exposure assessment of patulin in apple products was calculated for different population groups (i.e. infant, children, adults and elderly), taking into account the body weight, the daily consumption of apple juice and the patulin content. Due to a great variability observed of body weight and the consumption data of apple juices for the same population group, comparisons of the results obtained by different authors about the patulin intake in apple juices have been difficult.

The Joint Expert Committee on Food Additives (JEFCA), a scientific advisory body of the World Health Organization (WHO) and the Food and Agriculture Organization (FAO), allocated a provisional maximum tolerable daily intake (PMTDI) of 0.4 μg/kg body weight /day for patulin based on reproduction toxicity and carcinogenicity studies. In these studies, a NOEL of 43 μg/kg body weight/day was established and then a safety factor of 100 was applied (WHO, 1995).

Infants and young children are considered to be more susceptible to all the toxins than adults because of their lower body weight, higher metabolic rate, lower ability to detoxify, and incomplete development of some organs and tissues [43]. All studies undertaken in Europe showed that, infant and children were the main group exposed to patulin, in comparison with adults. Our results (Table 3) were also accordance to that. Consequently, they represent the highest risk group because they are the most susceptible, but also because their large dietary intake of apple-based foods.

The patulin intake derived from consumption of organic apple juices were in general higher than from conventional ones. Baert [41] reported for Belgium children a higher patulin intake for organic juice (0.019 μg/kg bw/day) than conventional one (0.009 μg/kg bw/day). Piemontese [23] compared four population groups (children, adolescents, adults and elderly), in all cases, the estimated daily intake derived from consumption of organic

Table 3. Exposure of the Catalonian population to patulin though apple juice consumption.

Population group (age)	Body weight (kg)	Consumption mL/day or kg/day	Apple juice characteristics	Patulin intake (µg/kg bw/day)	Percentage of the PMTDI[a]
Infants and young children (0-3)	12	20.4	Conventional	0.002	0.5
			Organic	0.016	4.0
Children (4-18)	40	64.5	Conventional	0.002	0.5
			Organic	0.015	3.8
Adults (19-66)	70	63.9	Conventional	0.001	0.3
			Organic	0.008	2.0

[a]Provisional Maximal Tolerable Daily Intake (PMTDI) 400 ng/kg bw; bw= body weight

apple juices was higher than from conventional. However, another Italian study [25] did not show this result.

Our results (Table 3) showed a highest patulin intake for organic apple juice, 0.016 µg/kg bw/day, 0.015 µg/kg bw/day, 0.008 µg/kg bw/day (infants, young children and adults respectively). Regarding to infants and young children, the daily intake of 20.4 mL of organic apple juices contributed to 4 % of the PMTDI. Considering the worse scenario for infants and young children, which implies a consumption of 200mL per day of the most contaminated brand of apple juice, the patulin intake was 0.223 ìg/kg, which represents more than a 50% of PMTDI. This suggests that in some cases, the intake of patulin could be close to PMTDI.

In summary, although the intake of patulin derived from consumption of organic apple juice were higher than that derived from conventional apple juice, all the estimated intakes of patulin calculated from the data of the studies presented in Tables 2 and 3 are below the PMTDI of 400 ng/kg bw proposed by JECFA and endorsed by the Scientific Committee of Food (SCF).

4. Conclusion

In recent years, several surveys have been conducted in Europe in order to analyse patulin levels in organic and conventional apple juices and to determine the associated risk for human health. Although the incidence and

levels of patulin were, in general, higher in organic than in conventional food, patulin intake in the population groups studied does not represent a health concern. All the estimated intakes of patulin calculated from the data of the studies presented, including our study, are below the PMTDI. However, there are vulnerable groups, such as infant and children, with higher risk associated to the consumption of these apple products.

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9. Current directions in DNA gel particles

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Abstract. A general understanding of interactions between DNA and oppositely charged compounds forms the basis for developing novel DNA-based materials, including gel particles. The association strength, which is altered by varying the chemical structure of the cationic cosolute, determines the spatial homogeneity of the gelation process, creating DNA reservoir devices and DNA matrix devices that can be designed to release either single- (ssDNA) or double-stranded (dsDNA) DNA. This paper reviews the preparation of DNA gel particles using surfactants, proteins and polysaccharides. Particle morphology, swelling/dissolution behaviour, degree of DNA entrapment and DNA release responses as a function of the nature of the cationic agent used are discussed. Current directions in the haemocompatible and cytotoxic characterization of these DNA gel particles have been also included.

Abbreviations

ALA : arginine-N-lauroyl amide dihydrochloride
CHIT : chitosan

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CTAB : cetyltrimethylammonium bromide
DDAB : didodecyldimethylammonium bromide
DTAB : dodecyltrimethylammonium bromide
DTAC : dodecyltrimethylammonium chloride
DTATf : dodecyltrimethylammonium trifluoromethane sulfonate
LAM : N^{α}-lauroyl-arginine-methyl ester hydrochloride
LS : lisozyme
MTT : 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PS : protamine sulfate

Introduction

The idea of gene therapy is to transfer genetic material into the cells to cure diseases through the expression of certain proteins. Despite significant advances in the past couple of decades, gene therapy is still in the clinical trial stage, mainly due to the lack of safe and efficient delivery vehicles for therapeutic nucleic acids. Deoxyribonucleic acid (DNA) is a negatively charged biomacromolecule that is subject to degradation in the bloodstream by endogenous nucleases [1]. Moreover, it is too large to cross the cellular membranes. A number of strategies are applied to limit DNA degradation, such as complexation with cationic species. DNA is a highly charged polyelectrolyte and therefore associates strongly with any oppositely charged cosolute, including simple ions, polymers, proteins, surfactants, lipids and other bioparticles. The interactions of cationic cosolutes with DNA have been extensively studied [2]. In general, strong associative phase separation is observed. The driving force for this strong association is electrostatic interaction between the two components. This is given by entropic increase due to the release of the respective counter ions.

A general understanding of the interactions between DNA and oppositely charged agents, and in particular the phase behaviour, has provided the basis for developing novel DNA-based materials, including gels, membranes and gel particles [2-4]. We have recently prepared novel DNA gel particles based on associative phase separation and interfacial diffusion. The polyelectrolyte of interest, DNA, is located in the core of these particles, while the complex between oppositely charged polyelectrolytes forms the corresponding shell. By mixing solutions of either single- (ssDNA) or double-stranded (dsDNA) DNA with solutions of different cationic agents, such as surfactants, proteins and polysaccharides, the possibility of forming DNA gel particles, without adding any kind of cross-linker or organic solvent, has been confirmed. Table 1 summarizes the characteristics of the different DNA gel particle systems. The association strength, which is altered by varying the chemical structure of the

Table 1. Characteristics of the obtained DNA gel particles (see abbreviation list at the end of the chapter).

CATIONIC AGENT	dsDNA	ssDNA	Reference
SINGLE-TAIL SURFACTANTS			
CTAB	x	x	[5,6]
DTAX	x	x	[7]
ALA, LAM	x	x	[8]
DOUBLE-TAIL SURFACTANTS			
DDAB	x	x	[9]
PROTEINS			
LS, PS	x		[5, 10]
POLYSACCHARIDES			
CHIT	x		[11]
REVIEW			[12]

cationic agent, enables the spatial homogeneity of the gelation process to be controlled to produce either a homogeneous DNA matrix or different DNA reservoir devices. These can represent a ‘‘bridge’’ for potential applications in the controlled encapsulation and release of ssDNA and dsDNA, with clear-cut differences in the mechanism. In the following sections, we describe the influence of the nature of the cationic agent, i.e. surfactant, protein or polysaccharide, on the degree of DNA entrapment, particle morphology, swelling/dissolution behaviour, and DNA release responses.

1. Degree of DNA entrapment

It is of major interest to characterize the degree of DNA entrapment on the DNA gel particles. The degree of DNA entrapment can be expressed as a function of the loading efficiency (LE) and loading capacity (LC) values. LE measures the amount of DNA that is included in the particles with respect to the total DNA, during particle formation. LC measures the amount of DNA entrapped inside the particles as a function of their weight. Characteristics of DNA gel particles, which are formed using surfactants, proteins, and polysaccharides as cationic compounds, are summarized in Figure 1. All values were measured in triplicate and are given as average and standard deviation

Except in the case of the double-tail surfactant DDAB, the LE values were always higher than 99%, which confirms the effectiveness of DNA entrapment in these cationic solutions (Fig. 1a). However, the entrapped

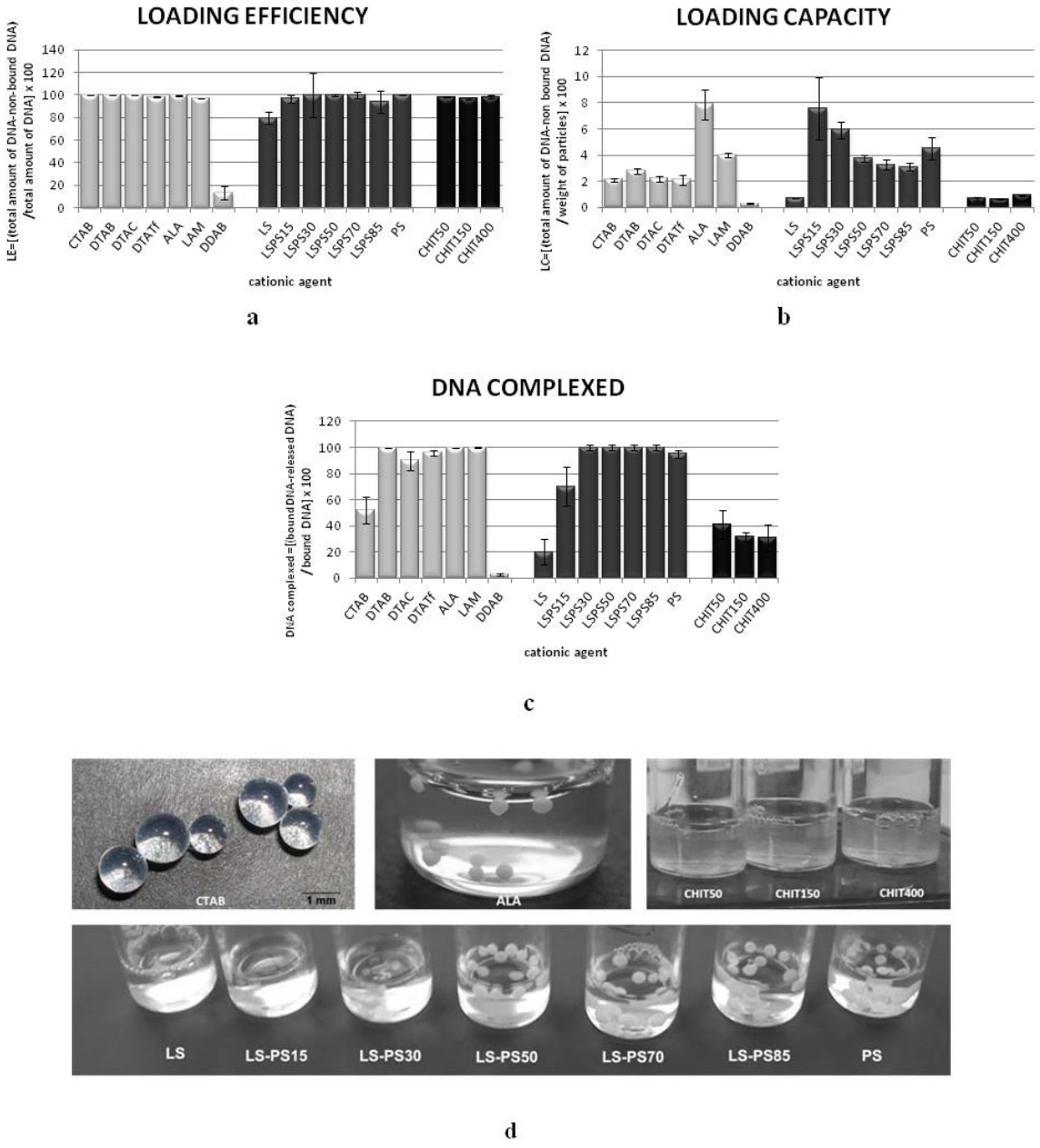


Figure 1. Characterization of the DNA gel particles with respect to DNA loading efficiency (a), loading capacity (b) and DNA complexed (c), as a function of the cationic compound (surfactants: light grey bars; proteins: dark grey bars; polysaccharides: black bars). Representative images of the obtained DNA gel particles showing translucent or opaque particles as a consequence of the characteristics of the gelation process (d). Adapted from references [5, 8,10].

DNA, as a function of the weight of the particles (LC values), depends on the cationic compound used (Fig 1b). The LC values obtained for surfactant-DNA gel particles depends on the hydrophilic and hydrophobic contributions. For the same type of polar head (CTAB and DTAB structures), the

hydrophobic contribution did not have a strong influence on the observed LC value [6, 7]. Identical LC values were obtained when DNA gel particles were prepared with surfactants that only differ in counter ion structure (DTAB, DTAC, DTATf) [7]. However, for the same hydrophobic chain length (DTAB, ALA and LAM structures), there is a clear effect of the number of charges on the polar head. Whereas DTAB and LAM showed one positive charge on the polar head, the ALA structure showed two positive charges. Accordingly, higher the number of charges, higher the LC values [8]. When the DTAB and DDAB structures are compared, we can conclude that higher number of hydrophobic chains on the surfactant structure contributes negatively to the LC values [9]. In the case of protein-DNA gel particles, the lowest LC value (0.7%) was obtained in particles formed with pure LS. Interestingly, highest LC value was obtained for particles containing the smallest amount of PS [10]. Molecular weight of the polysaccharide structure did not have a clear effect on the LC values [11].

An indication of the structural characteristics of these DNA gel particles can be deduced from the amount of DNA released, when particles' breakup is mechanically promoted. The percentages of DNA complexed were calculated and are summarized in Fig. 1c. Complexed DNA is related to the amounts of DNA in the supernatant solutions and the skins derived from the particles, after particles were magnetically stirred overnight. In the case of surfactant-DNA gel particles, the percentages of complexed DNA suggest that, when surfactant structures are used with twelve carbon atoms in the hydrophobic chain (DTAB, DTAC, DTATf, ALA, LAM), most of the DNA is complexed during particle formation [7, 8]. More limited complexation has been obtained by increasing, either the alkyl chain length (CTAB), or the number of alkyl chains in the molecule (DDAB) [6, 9]. In the case of the protein-DNA gel particles, the amount of complexed DNA increases progressively in the presence of the protein protamine sulphate [10]. The amount of complexed DNA in the case of chitosan-DNA gel particles seems to decrease when the molecular weight of the polysaccharide is increased [11].

This distribution could be correlated with differences in the gelation process during particle formation. Homogeneous gelation can lead to homogeneous structures (solid particles), whereas a more inhomogeneous gelation process forms core-shell structures [13]. In the present study, the model distribution of DNA in the particles was supported by visual inspection, since translucent core-shell particles and opaque condensed particles were found (Fig. 1d).

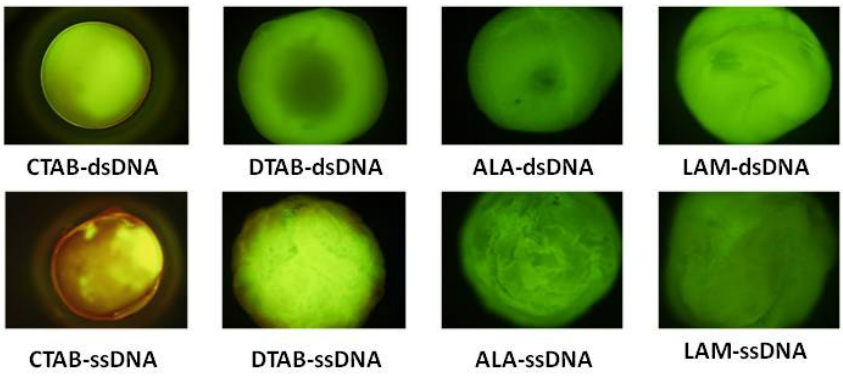
2. Morphological characterization of the DNA gel particles

The secondary structure of the DNA molecules in the gels was studied by fluorescence microscopy (FM) using acridine orange staining. Acridine orange (AO) has been used to label nucleic acids in solution and in intact

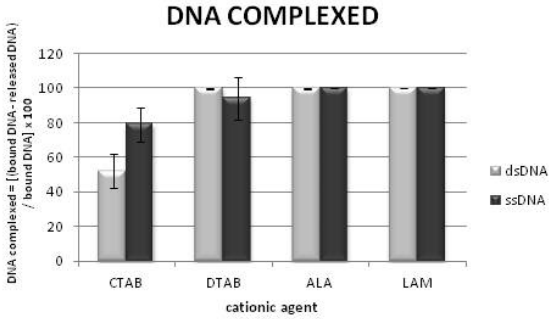
cells [14-17]. In the case of AO-dsDNA, the fluorescence emission shows a maximum around 530 nm, in the green spectra. The association with ssDNA shows a maximum around 640 nm, in the red spectra.

Based on the observation of green or red emission, AO was used to differentiate between native, double-stranded DNA, and denatured, single-stranded DNA, in the gel particles. Fig. 2a shows fluorescence micrographs of individual particles of the surfactant-dsDNA systems.

FM studies have revealed that the formation of particles with double-stranded DNA is carried out with conservation of the secondary structure of the DNA. However, in the case of particles formed with denatured DNA, green emission is also observed, except in the case of CTAB-ssDNA gel particles. The absence of red emission in the particles containing denatured DNA suggests that the accessibility of free DNA to the dye is hindered. This observation is consistent with our data on DNA distribution (Fig. 2b). The percentage of DNA released was less than 0.1%, which confirms the total complexation of the DNA. However, when CTAB was used, the amount of ssDNA released reached 20%, making its detection possible in fluorescence microscopy studies.



a



b

Figure 2. Fluorescence microscopy micrographs of individual surfactant-DNA gel particles in the presence of the DNA selective dye AO (a). Complexed DNA is related to the amounts of DNA in the supernatant solutions and the skins derived from the particles, after particles were magnetically stirred overnight (b) Adapted from references [6,8,9].

Scanning electron microscopy imaging was carried out to establish possible differences in the morphologies between the different particles. Figure 3 shows representative images of CTAB and DTAB surfactants. Clear similarities were found in the outer surface morphology between these four formulations. However, the surface of the inner structure revealed a different structure. Large pores and channel-like structures were found in the inner surface of particles formed with CTAB. However, the structure of the particles formed with DTAB revealed a more compact structure. The structures obtained seem to confirm the degree of complexation between these two surfactants and DNA (see Fig. 2b), which increases the shell section of the obtained particles.

Similar experiments were carried out on particles formed with the double-tail surfactant DDAB [9]. In this case, spherical domains formed that were visible on the surface of these DDAB-DNA particles (Fig. 4a). The nature of these domains was studied using the hydrophobic dye Nile Red (NR) (Fig. 4b) [18]. The fluorescence emission of NR, in the presence of DDAB-DNA gel particles, was nearly identical to that recorded for DDAB vesicles. This indicates that the observed spherical domains on the surface of the DDAB-DNA gel particles are composed of hydrophobic layers of the surfactant. The examination of the DDAB-DNA gel particles with the DNA-selective dye AO revealed that the DNA is also included in these domains. Differences in the reorganization of DNA were found as a function of the secondary structure.

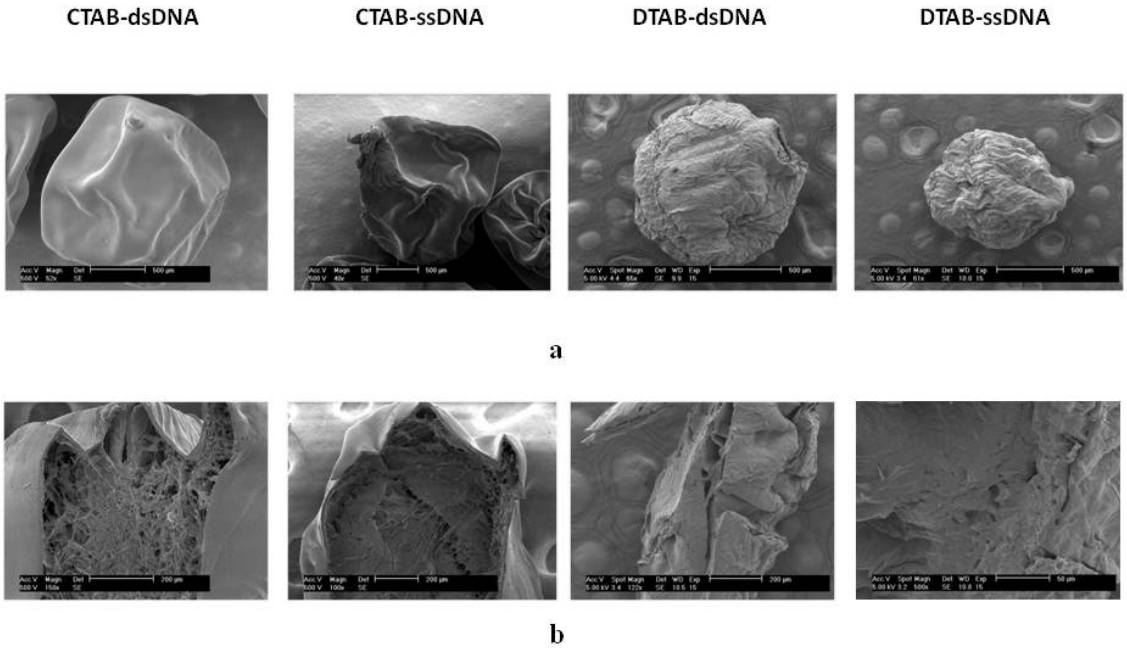


Figure 3. Scanning electron micrographs of individual CTAB–DNA and DTAB-DNA gel particles: outer surface (a) and cross-sections showing both the outer and inner surfaces (b). Adapted from references [5,9].

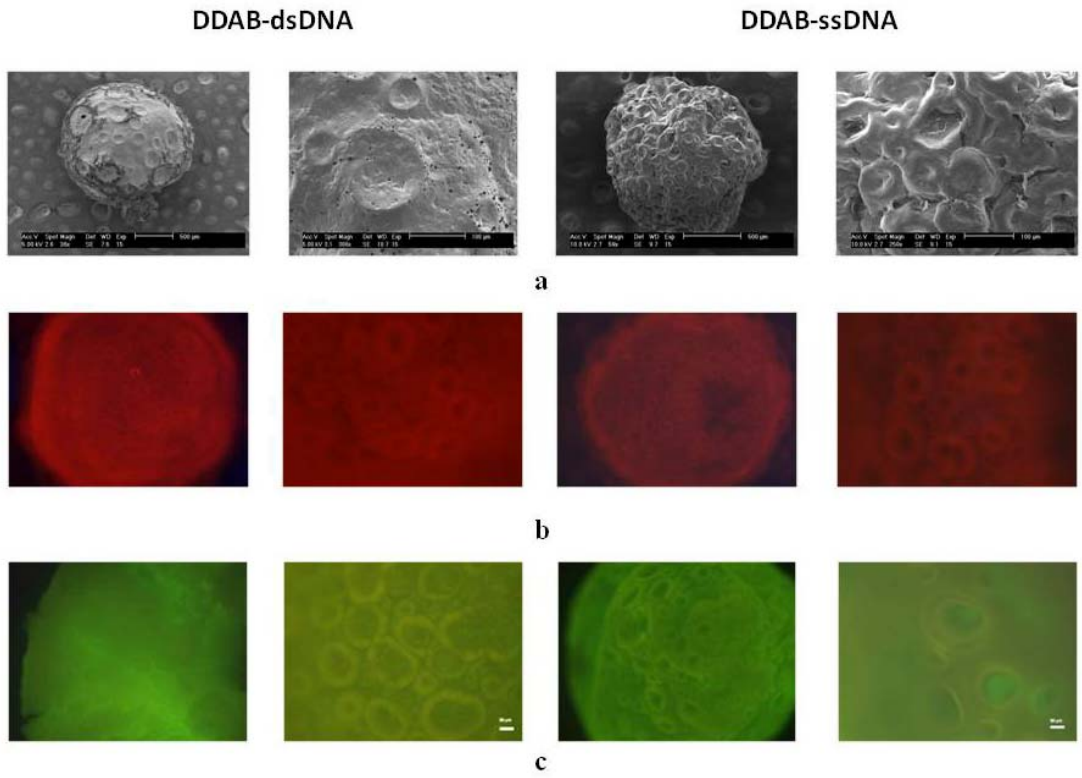


Figure 4. Scanning electron micrographs (a) and fluorescence microscopy using Nile Red (b) and Acridine orange (c) dyes of individual DDAB-dsDNA and DDAB-ssDNA gel particles. Adapted from reference [9].

In the case of particles formed with native DNA, the observed vesicular domains seem to have grown by fusion of several vesicles, adopting a near-spherical shape. However, the greater thickness of the vesicular domains found in the DDAB–ssDNA particles suggests that the reorganization of DDAB vesicles in the presence of denatured DNA takes place with the subsequent formation of multilamellar complexes. Although these DDAB–DNA particles were prepared at the same DNA/DDAB ratio, the results indicate that differences in local DNA concentration or some kind of inaccessibility of one of the components can be significant. FM images at higher magnification also support these differences.

3. Swelling/dissolution behaviour and kinetics of DNA release

Gels are considered to have great potential as drug reservoirs. Loaded drugs can be released by diffusion from the gels or by the erosion of them. When the DNA gel particles are inserted into a certain medium, different responses occur: swelling or deswelling, dissolution, and release of DNA.

In the case of surfactant-DNA gel particles, the extension of the swelling process depends on both the surfactant and the nucleic acid structures (Fig 5a). For the same hydrophilic contribution (CTAB and DTAB), the decrease in the number of carbon atoms in the hydrophobic chain contributes negatively to the swelling extension. So, when CTAB-DNA gel particles were placed in pH 7.6 10 mM Tris HCl buffer, water was taken up from the medium and swelling could be observed. The swelling continued during the entire time interval studied (1,200 h) [6]. However, DTAB–DNA particles showed an initial swelling and then dissolved completely after 48 h. DTAB-DNA gel particles have the largest relative weight (RW=4–6, depending on the secondary structure of the nucleic acid) [7].

Generally, the release pattern resembles that observed in the swelling/dissolution profiles (Fig. 6a). Thus, CTAB-DNA particles placed in pH 7.6 10 mM Tris HCl buffer showed no initial burst release: in the first 24 h, only 1.6, 2.0 and 3.3% of DNA was released, respectively. After 1,200 h, 69 and 40% of DNA was released from the particles containing dsDNA and ssDNA, respectively [6]. Nevertheless, DTAB–DNA particles exhibited fast release behaviour by a dissolution mechanism. The corresponding half-lives of DTAB–dsDNA and DTAB–ssDNA are 4 and 8 h, respectively. After 24 h, more than 97% of the bound DNA was released [7].

For the same hydrophobic contribution (ALA and LAM derivatives), the swelling/dissolution behaviour can be modulated by modification of the type and number of positive charges on the polar head [8]. Particles containing ALA exhibited the largest (relative weight ratio, RW 2-2.5) and the longest (more than 1,300 h) swelling process. Particles containing LAM swelled (RW 1.5-2.0 using the maximum points as estimate) for up to 10 or 200 h, as a function of the secondary structure of the polyelectrolyte, and then started to shrink. The results suggest that the stability of the gel particles is given mainly by the electrostatic interaction between DNA and the oppositely charged surfactant. More stable particles were obtained for ALA than for LAM, probably due to its double charge. In addition, in the latter case, the stability was higher when denatured DNA was used. Thus, LAM-DNA particles exhibited faster release than ALA-DNA particles (Fig. 6a). Complete release from LAM-dsDNA particles occurred after 400 h; whereas complete DNA release from LAM-ssDNA occurred after 800 h. When the formulation contained ALA, the DNA release was slower. Complete DNA release was only achieved after 1800 h. With respect to the observed differences between ssDNA and dsDNA release, the results agree with our previous studies on surfactant and protein systems, which showed a stronger interaction with ssDNA than with dsDNA [5,6,]. The rate and the final cumulative DNA release depend on its secondary structure. This strongly

indicates the important role of hydrophobic interaction in DNA when the bases are more exposed, as in the case of ssDNA.

Furthermore, the increase in the number of alkyl chains (DDAB) contributes positively to the stabilization of the particles [9]. DDAB-DNA particles absorbed an amount of water that was twice the initial mass (relative weight, RW, of 2, using the maximum points as an estimate). These particles had returned to the original particle weight by the end of the experiment (1,500 h). In the case of DNA gel particles formed using the double-tail surfactant DDAB, an initial burst release was observed (Fig 6a). The duration of this burst release (24 h) was independent of the secondary structure of the nucleic acid used, but not of the amount released. The amount of DNA released in the first 24 h was 44% and 15% for DDAB–dsDNA and DDAB–ssDNA particles, respectively. The presence of this burst suggests that some DNA is not encapsulated, or DNA is bound weakly on the surface of the particles. From 24 to 600 h, a plateau was observed in the cumulative DNA release. After that, particles placed in the buffer solution showed a change in release kinetics. A linear cumulative release was observed until the end of the experiment (1,500 h). The amount of DNA released from DDAB–dsDNA and DDAB–ssDNA was 63 and 34%, respectively.

Concerning protein-DNA gel particles, LS-DNA gel particles lost weight rapidly and extensively (Fig. 5b) [10]. In the case of PS-DNA particles, the largest relative weight ratio was observed (RW>5). For the high LS/PS ratio, particles absorbed a water amount of 2-3 times the initial mass (relative weight ratio, RW, of 2-3) during the swelling process. With a decrease in the LS/PS ratio, more moderate absorption of water was observed (RW=1-2). When the particles contained PS, there was a common trend in the swelling profiles, in which initial swelling was visible before the particle started to dissolve. The initial period in the swollen state, before dissolution takes place, was independent of the PS content and lasted approximately 100 h (using the maximum of the first peak as an estimate). Then, a short period of stabilization was observed after a new, more limited, maximum appeared (located around 400 h). Thereafter, the RW value became approximately constant, with two exceptions. For PS-DNA particles, RW increased with time, while for the LS-PS15 system, nonmonotonic behaviour was observed.

LS-DNA particles exhibited fast burst release behaviour by a dissolution mechanism (Fig. 6b). After 24 h, 84% of the bound DNA was released. When the formulation contained PS, the initial burst release was absent. The percentage of DNA released in the dissolution media, after 24 h, varied from 0.4 to 1.0% for protein mixed systems. The absence of a burst effect suggests that minimal amounts of unencapsulated DNA are present on the surface of the particles after their formation. For particles containing both proteins, the

profiles showed slower DNA release than in the pure systems. The release rates remained almost constant in the case of particles formed at a high LS/PS ratio (LS-PS15 and LS-PS30). However, with a decrease in the LS/PS ratio, a sudden acceleration of the release was observed after ≈ 400 h. We can assume that complete hydration of the core in our particles could occur after 400 h, taking into account the presence of the maximum RW values in the swelling-dissolution experiments (Fig. 5b). This matrix swelling behaviour could determine the change in the rate of DNA release, which became dependent on the LS/PS ratio. In addition, the final release percentage was largely dependent on the LS/PS ratio. As indicated by the arrow in Figure 5, the formulations with the lowest PS content released only a small percentage of the DNA present in the particles (<20%), but this percentage increased with PS content to attain ca. 80% for the LS-PS85 formulation.

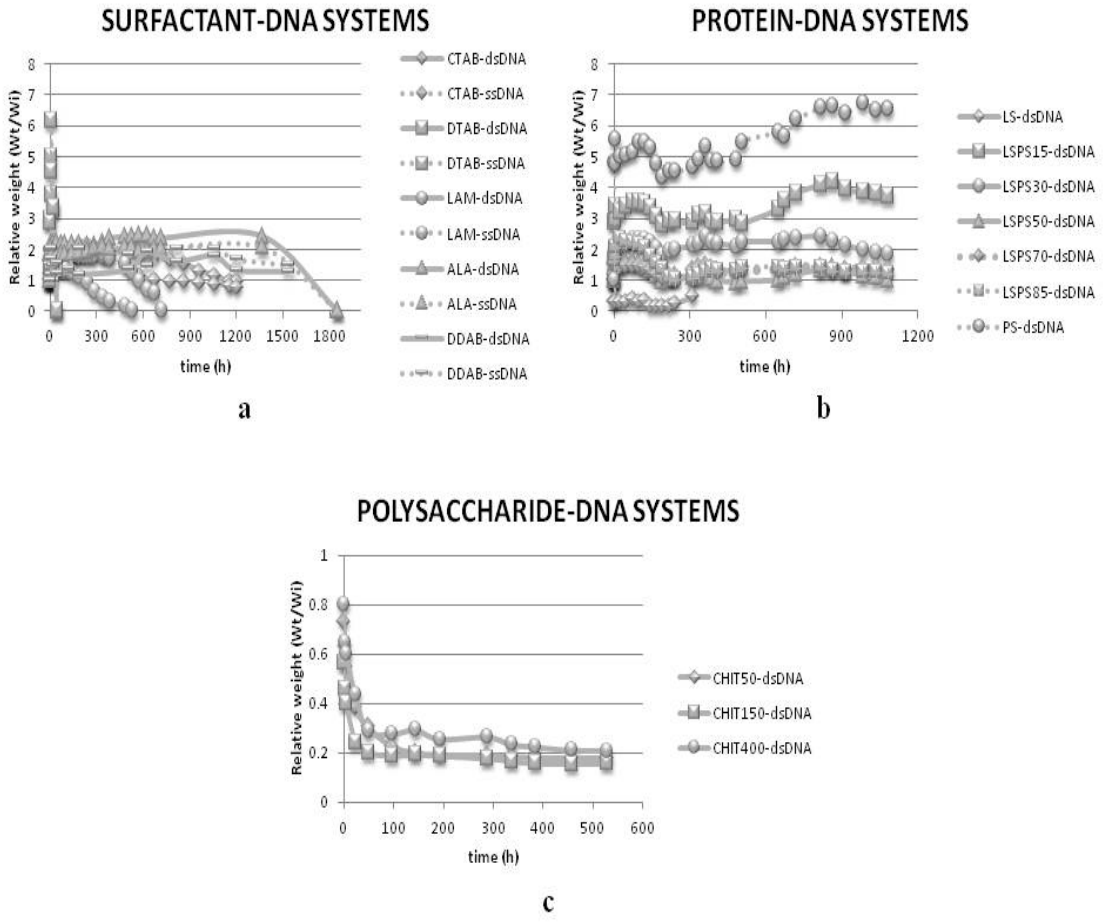


Figure 5. Time-dependent changes in relative weight RW measurements performed on surfactant-DNA (a), protein-DNA (b) and polysaccharide-DNA (c) gel particles after exposure to pH 7.6 10 mM Tris HCl buffer solutions. Where W_i stands for the initial weight of the particles and W_t for the weight of the particles at time t. Adapted from references [10,11].

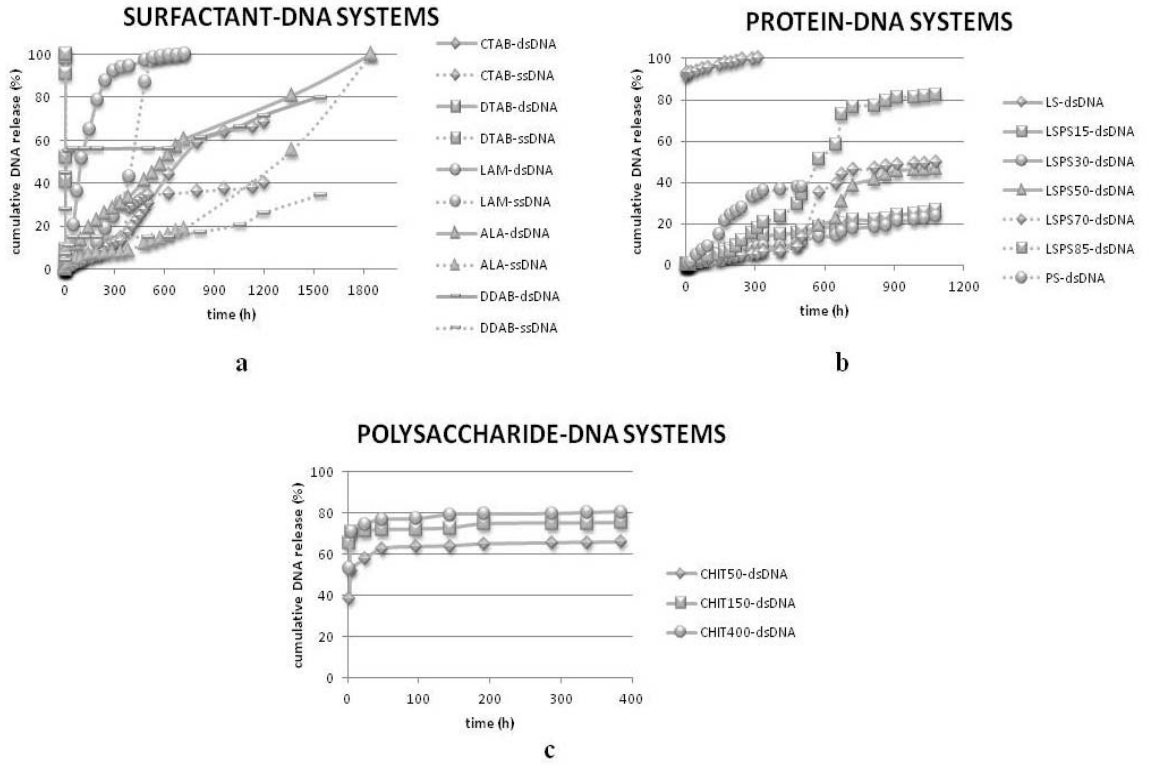


Figure 6. Time-dependent changes in DNA release measurements performed on surfactant-DNA (a), protein-DNA (b) and polysaccharide-DNA (c) gel particles, after exposure to pH 7.6 10 mM Tris HCl buffer solutions. Adapted from references [10,11].

The determination of the kinetics of swelling and dissolution behaviour demonstrated that chitosan-DNA gel particles lost weight rapidly and extensively (Fig. 5c). The molecular weight of chitosan has a significant effect on the encapsulation of DNA and on the *in vitro* release properties. The release of DNA from the different particles is illustrated in Fig. 6c. Generally, DNA release rates in the initial period were high in all cases. In the first 24 hours, 57%, 71%, and 74% of DNA were released from the particles containing low, medium and high molecular weight chitosan, respectively [11].

4. Determination of *in vitro* biocompatibility

The safety evaluation of new products or ingredients destined for human use is crucial prior to exposure. Rapid, sensitive and reliable bioassays are required to examine the toxicity of these substances. Established cell lines areuseful alternative test systems for this kind of toxicological studies [19]. However, they must be chosen with care with regard to their origin [20]. Cytotoxicity assays are among the most common *in vitro* endpoints used to predict the potential toxicity of a substance in a cell culture [21]. It is

essential to understand the interactions of the DNA-based particles with cells *in vitro* to improve their behaviour *in vivo*.

Nano-sized materials (NMs) have a high potential in technical and medical applications provided they are not toxic. Despite the significant scientific interest and promising potential, the safety of nanoparticulate systems remains a growing concern, considering that biological applications of nanoparticles could lead to unpredictable effects. The prediction of toxicity is difficult, but cytotoxicity screening, which is routinely used in drug screening, gives a good indication of potential adverse effects in cells. These screening assays are also used for NMs. As a general rule, nano-sized materials show higher reactivity than bulk materials of the same composition. Therefore, toxicity data must be interpreted in the context of the physicochemical characteristics of the NM [22].

Size, surface charge, and hydrophobicity interact in complex ways and have a pronounced influence on biocompatibility. Aggregation of NMs in physiological fluids is often observed. Based on the assumption that a decrease in cellular vitality reduces physiological function, cellular products and cell number, cytotoxicity screening assays that measure enzymatic activities or cell products have been carried out. These assays perform reliably with chemical compounds but can produce false results by interference with NMs. Most examples have been published for the MTT assay, which is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide by cellular dehydrogenases. NMs interfere with the assay by light absorbance, reduction of the tetrazolium salt, and binding of the formazan salt [23]. Interactions of NMs with assays include interference by adsorption of dyes [24], absorbance [25], fluorescence [26], binding of proteins [27], dye degradation [28], and dye formation [24] among others. The most important conclusion drawn from these studies was that more than one cytotoxicity assay should be used to evaluate NMs.

No specific cytotoxicity screening assays and no specific cell lines are fixed to study the effect of NMs. Therefore, a variety of cell lines are being used for cytotoxicity screening. Usually, the concentration that has a half-maximal effect on the decrease of viability is considered as the toxic concentration (TC_{50}), effective concentration (EC_{50}), or inhibitory concentration (IC_{50}).

Although no prominent variation between species have been reported for conventional chemicals, NMs may react differently. An investigation of the cytotoxicity of cationic polystyrene nanospheres in five cell lines was reported. It was found that despite uptake in all cell lines, the nanospheres only affected cytotoxicity in two lines [29]. The different reaction of the cell lines may be due to their specific characteristics. Potential parameters that

could influence the results of the cytotoxicity screening of NMs include the phagocytic capacity of the cells. Other cellular parameters, like doubling time, size, embryonic origin etc., could also play their role. Knowledge about such potential differences can help in the design of cytotoxicity testing of new NMs and in explaining cytotoxicity data that varies between laboratories.

One of the most common non-epithelial cell lines used in short- and long-term nanotoxicological *in vitro* studies on: cytotoxicity, biocompatibility, or mechanisms of cellular uptake of nanoparticles, are the 3T3 fibroblasts. These are readily available, undergo contact inhibition, and are more closely representative of a physiologic model than cancer cell lines, for example [30]. Our group studies have demonstrated that the cytotoxicity of cationic nanovesicles differed depending on the cell lines as well as the *in vitro* endpoint that was measured. This shows that the selected cell type and assay can affect the final outcome, as indicated by other authors [22, 24]. Overall, our findings showed that nanovesicle composition plays a primary role in underlying toxicity. The cytotoxic responses of the nanovesicles varied especially as a function of the cationic charge position on the amphiphile that was included in them. Furthermore, the surfactant with the highest hydrophobicity tends to enhance the toxic potential of the formulations. All these findings suggest that differential toxicity according to vesicle composition could be an important concept when new nanomaterials are developed for biomedical applications. In conclusion, the combination of all assays used in the present study offers an in-depth and comprehensive evaluation of the potentially toxic effects of nanomaterials [31].

Another problem involved in the determination of the cytotoxic effect of nanomaterials is interference with the medium and possible changes in size, when they are incorporated into the cell culture medium. The adequate size characterization of nanomaterials is a prerequisite for meaningful outcomes of nanotoxicity studies [32]. Indeed, in contrast to synthetically produced nanoparticles, nanovesicles are much less homogeneous in size and composition. Thus, aggregation of nanovesicles and size distribution will affect the outcome of toxicity tests dramatically, as larger particles may settle faster in cell culture, and are less available to cells than smaller ones, that remain in suspension longer and are thus available for incorporation via endocytosis [33]. Obviously, there are numerous methods for the characterization of nanomaterials [34], but no single method will permit a description of nanovesicle characteristics that can support an improved interpretation of the *in vitro* effect data. In studies of the toxicity of nanovesicles, cytotoxicity assays should be performed for the nanovesicles and for different components such as the cationic surfactants involved in

these particles. Our group has evaluated the cytotoxicity of different cationic surfactants [35,36].

Another important feature in the development of nanoparticulate systems for parenteral administration is to determine their ability to cause haemolysis by interaction with the cell membrane. The potential uses of colloidal self-assemblies as drug delivery systems make haemolysis evaluation very important. To this end, we examined this interaction by using erythrocytes as a model biological membrane system, since erythrocytes have been used as a suitable model for studying the interaction of amphiphiles with biological membranes [7, 37-39] Most *in vitro* studies of surfactant-induced haemolysis evaluate the percentage of haemolysis by spectrophotometry, to detect plasma-free haemoglobin derivatives after incubating surfactant solutions with blood and then separating undamaged cells by centrifugation. However, in the case of particles, the interpretation of the results of these studies is complicated, due to the variability of experimental approaches and a lack of universally accepted criteria for determining test-result validity.

Studies of our group carried out with surfactant-DNA gel particles have demonstrated that the amount of DNA that is released and the haemolytic response induced by surfactant-DNA gel particles are strongly dependent on both the structure of the counter ion in the surfactant and the secondary structure of the DNA [7]. One drawback of these surfactant-DNA gel particles, in toxicological terms, is the need for a cationic surfactant, which may cause some cellular damage. However, our results indicate that the effect of the surfactant can be modulated when administered in the DNA system, unlike an aqueous solution. This modulation is due to the strong interaction between the surfactant and the biopolymer, which leads to very slow release of the surfactant from the vehicle.

The surfactant-DNA interaction reflects both the release of haemoglobin (degree of haemolysis) and the release of DNA into the media, as a consequence of the dissolution kinetics of the polyelectrolyte-surfactant complexes. Under the experimental conditions in which the haemolysis studies took place, the amount of dsDNA that is released at the end of the experiment (180 min) reaches 100 mg/mL. However, with particles prepared with denatured DNA, only 10% of this amount is released into the media. These differences are supported by visual inspection: surfactant–dsDNA particles are completely dissolved at the end of the experiment, whereas surfactant–ssDNA particles are still present after 180 min. With respect to the surfactant structure, the haemolysis response found in these DNA gel particles can be correlated with differences in the apparent degree of counter ion dissociation in these surfactants from the corresponding micelles.

5. Conclusion

A general understanding of interactions between DNA and oppositely charged agents provides a basis for developing novel DNA gel particles. When the DNA gel particles are inserted in a medium, different responses occur: swelling or deswelling, dissolution, and DNA release. One drawback of the DNA gel particles, in toxicological terms, is the need for a cationic compound, which may cause some cell damage. However, our results indicate that the effect of the cationic agent can be modulated when administered in a DNA gel system, rather than in an aqueous solution. While toxicity certainly applies for most classical surfactants, we are engaged in work on haemocompatible and cytotoxic assessments of DNA gel particles prepared with cationic compounds with much improved intrinsic biocompatibility. These include surfactants with the cationic functionality based on an amino acid [8], polysaccharides [11] and proteins [10]. There is special interest in decreasing the size of these DNA gel particles [40], which is a prerequisite for cellular uptake and internalization, and subsequent DNA delivery and transfection.

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10. Evaluation of reclamation technologies for wastewater reuse

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Abstract. Reclamation and reuse of wastewater require the use of tools that minimize risks to health and natural ecosystems. There are various types of such tools, among which HACCP (hazard analysis and critical control points) and barrier systems are gaining importance. The research reported here aims to determine and evaluate the most efficient combinations of different treatment systems—barriers—for the reclamation of secondary effluents from urban sewage treatment plants, and for obtaining water of sufficient quality for reuse in accordance with existing legislation, in which water disinfection has become one of the keys to compliance. Several conventional and non-conventional reclamation technologies are evaluated. The results lead us to recommend treatment lines for the different reclaimed water uses established in the Spanish legislation.

Introduction

Reclaimed wastewater can be used to meet part of the demand for water in those areas where the natural supply is insufficient due to the climatology. Reclaimed water resources must be seen in the context of integrated water resource management, mainly as a replacement resource in accordance with

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the quality required for each use. In this regard, it should be noted that such resources are particularly suitable for meeting that part of the demand which does not require water of high sanitary quality [1].

As in other Mediterranean countries, wastewater reuse in Spain is emerging as a viable alternative with an extremely promising future due to the many benefits it presents, such as increased water resources, reduced wastewater discharge into the environment and decreased negative environmental impacts arising from water extraction from the natural environment. However, it also presents some drawbacks, such as risks to health, the need for investment and a reduction in flow rates available in certain sections of a watershed. At present, although the technologies currently available for wastewater reclamation have the capacity to achieve the quality required for reuse in different scenarios, they present several weaknesses, including uncertainly about the reliability of the reclamation processes and about the representativeness of the reclaimed water samples for analysis. Hence, there is a significant need for research, development and innovation aimed at defining and combining treatment systems, and for proactive management of the data obtained [2]. In Spain, the quality required of reclaimed water destined for various uses is established by Royal Decree (RD) 1620/2007 [3]. However, this RD does not constitute a definitive framework for wastewater reclamation and reuse, and has been the catalyst for constant debate about its relevance to the reality of Spain, the errors detected and the economic feasibility of its application, especially as regards the amount of analysis required. The RD is not in line with global trends aimed at overcoming strict adherence of the legislation to reference standards, and thus does not advocate calculations tailored to each situation [4,5,6].

However, even working within the established definition of hazard analysis and risk management, the need for research into the best treatments for wastewater reclamation and reuse still remains. The experimental study presented here formed part of a research project on reclamation and reuse of secondary effluents. The main objective of the project was to evaluate the efficiency of several treatment technologies that were combined in different wastewater reclamation treatment lines aimed at obtaining reclaimed water suitable for reuse in accordance with existing legislation.

1. Reclamation technologies

The study was carried out at a municipal WWTP (wastewater treatment plant) located on the Spanish Mediterranean coast. This plant is an activated sludge facility that has a theoretical flow of 2,400 m^3/day. Part of the secondary effluent was reclaimed by the facility's existing technologies,

which consisted of three pre-treatment systems before disinfection (ring filter, physical-chemical and infiltration-percolation) and three disinfection technologies (chlorine dioxide, peracetic acid and ultraviolet disinfection). All studies were conducted at pilot scale except for infiltration-percolation, and all possible combinations were considered, yielding a total of nine different treatment lines for analysis (Table 1). For the purposes of this study, a treatment line was defined as the combination of a filtration technology (pre-treatment technology) followed by a disinfection technology.

Table 1. Treatment lines evaluated.

Treatment line	Pre-treatment technology	Disinfection technology
1	Ring filter	Chlorine dioxide
2	Ring filter	Peracetic acid
3	Ring filter	Ultraviolet radiation
4	Physico-chemical	Chlorine dioxide
5	Physico-chemical	Peracetic acid
6	Physico-chemical	Ultraviolet radiation
7	Infiltration-percolation	Chlorine dioxide
8	Infiltration-percolation	Peracetic acid
9	Infiltration-percolation	Ultraviolet radiation

1.1. Ring filter

The ring filter (RF) is a filtration system consisting of modules formed by flat plastic rings with slots which are connected in series or in parallel. The extent to which the rings overlap determines a specific light path which in turn determines the particle size retained by the filter [7]. Decompression of the rings to be cleaned occurs in the washing process, and the main mechanism of contaminant removal is mechanical filtration.

The ring filter used was provided by Hidroglobal-Arkal, and consisted of a filtration module in parallel, with a total of four filters (Fig. 1). Each filter body consisted of flat plastic rings with 25 μm slot openings. The feed flow was 9 m^3/h.

Figure 1. View of the ring filter.

1.2. Physico-chemical system

Physical-chemical systems (PC) are based on the addition of coagulants and flocculants to a mixing tank, followed by settling and sand filtration. The main mechanisms of contaminant removal are coagulation-flocculation, sedimentation and mechanical filtration [7]. For the PC system used here, we employed an inorganic coagulant (poly aluminum PAX18), followed by lamellar settling and, at the end of the process, two sand filters for mechanical filtration. The feed flow was $8m^3/h$, and the pilot plant and necessary reagents were provided by Kemira (Fig. 2).

Figure 2. View of the physico-chemical system.

1.3. Infiltration-percolation

Infiltration-percolation (IP) is a non-conventional or extensive technology that can be defined as an aerobic treatment process with a fixed biomass (biofilm) in unsaturated fine granular media (sand) which uses sequential

feeding with a discontinuous input of organic matter, nutrients and oxygen, infiltrating wastewater in a controlled manner [8,9]. Infiltration-percolation uses a filter bed to which a biofilm is attached. The filter bed consists of sand with a particle size calibrated to achieve filter uniformity. The granulometry of the sand ensures rapid renewal of the gas phase, and permits retention of suspended solids and control of the percolation rate in relation to oxidation and disinfection kinetics. Grass is always present on the surface of the system in order to avoid preferential hydraulic flow paths.

IP systems are mainly based on the presence of a biofilm, which implies a carrier material (grains of sand) and organisms capable of forming a biofilm. The biofilm is mainly composed of bacteria, although other organisms such as protozoa and metazoa are present. These organisms form complex aggregates with extracellular polymers and metabolic compounds and minerals. Infiltration-percolation systems act to remove wastewater contaminants basically through mechanical filtration and biological oxidation [9]. The IP system used here was especially constructed for this project (Fig. 3) and consisted of a 1.50 m deep sand filter bed (98% particle size <1 mm in diameter, d10 0.28 mm, with a uniformity coefficient of 3.61) which provided a functional surface of 144.67 m^2 and had a nominal hydraulic loading capacity of 0.69 m/day. The feed flow was 6 m^3/h. Wastewater was fed into the system through evenly distributed subsurface drip irrigation lines.

Figure 3. View of the infiltration-percolation system.

1.4. Chlorine dioxide

Chlorine dioxide (ClO_2) is an effective oxidant used for phenol contaminated waters and for eliminating odor problems while disinfecting. This disinfectant does not react with ammonia or with bromine, and does not generate noticeable amounts of byproducts, although oxidized compounds

and ions such as iron, manganese, and nitrates may be generated [7]. Due to its instability, chlorine dioxide must be generated in situ. The pilot plant used in this project consisted of a Bellozon CDVa35 chlorine dioxide generator (ProMinent Gugal, SA), a homogenization tank of 1 m^3 and a shaker (Fig. 4).

The feed flow was of 1m^3/h, with a peak production of ClO_2 of 46 g/h. The chlorine dioxide production method consisted of mixing a sodium chloride solution with a hydrochloric acid solution, according to the following reaction:

$$5NaClO_2 + 4HCl \rightarrow 4ClO_2 + 5NaCl + 2H_2O$$

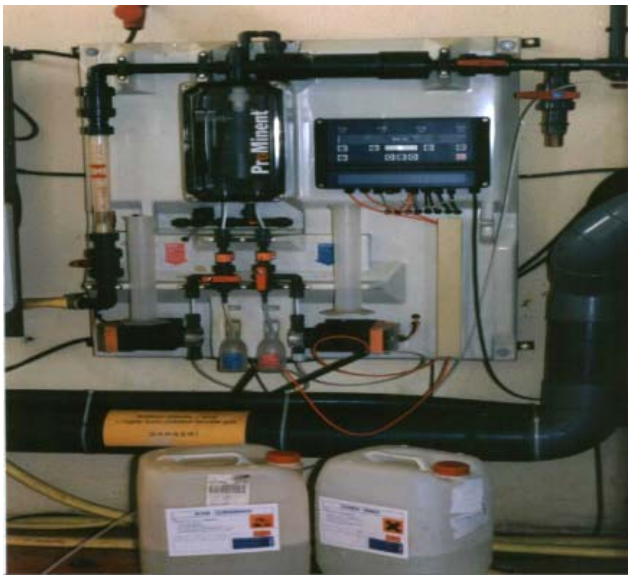

Figure 4. View of the chlorine dioxide generator.

1.5. Peracetic acid

Peracetic acid (PA) is a powerful oxidant and its disinfectant action is due to the damage caused at the lipoprotein membrane of the microorganisms, modifying the conveying action of the proteins leading to lysis [7]. Peracetic acid is chemically unstable and is produced from the reaction between hydrogen peroxide and acetic acid, according to the following reaction:

$$CH_3\text{-}COOH + H_2O_2 \rightarrow CH_3\text{-}COOOH + H_2O$$

The peracetic acid system used consisted of a stirred reactor which was fed with the wastewater to be treated and peracetic acid, which was dosed by a pump (ProMinent Gugal, SA.) (Fig. 5) fitted with a pressure valve to prevent fluctuations. The feed flow was 1m^3/h.

Figure 5. View of the peracetic acid system.

1.6. Ultraviolet radiation

The ultraviolet radiation (UV) system is a physical disinfection technology that acts on the nucleic acids and proteins of the microorganisms, deactivating them [7]. Normally, monochromatic radiation of 253.7 nm is used because it is considered the most effective wavelength as a germicide, although the possibility of bacterial reviviscence must be taken into account. The UV system used (UV3000PTP) was provided by Trojan (Fig. 6). The system had six low pressure lamps, was 1,626 mm long and was used with a feed flow of 5 m^3/h.

Figure 6. View of the peracetic acid system.

2. Evaluation of reclamation technology's efficiency

The doses and contact times of the disinfectants were established in preliminary tests, and varied according to the filtration process to which the secondary effluent was subjected, as indicated in Table 2.

The chemical parameters analyzed [10] included some of the parameters required by Spanish legislation, in addition to other parameters that permit a better assessment of disinfection effectiveness. The microbiological parameters analyzed consisted of two bacterial indicators, E. coli and total coliforms [10], and a virus indicator, somatic bacteriophages [11]. E. coli was selected as the bacterial indicator because it is used in Spanish and international reuse legislation. Total coliforms are used in more restrictive regulations, such as the legislation in the State of California, but there is a worldwide trend to stop using them because their origin is not only fecal and they can be found in natural environments. Each treatment line was evaluated independently for one year.

The secondary effluent was characterized by physical-chemical and microbiological parameters (Table 3).

According to the obtained results, the secondary effluent was very heterogeneous with large deviations, especially as regards COD, SS, and turbidity, parameters that are known to affect the performance of disinfection technologies. Also notable was the variation in dissolved oxygen content, which can affect the performance of infiltration-percolation systems.

The results obtained during evaluation of the outlet effluent from the pretreatment technologies are shown in Figure 7. There was a certain degree of variability in COD, suspended solids and turbidity at the outlet of the different pretreatment systems. According to the results, the pretreatment systems achieved homogenization of effluent quality and had the capacity to cope with peak loads of contaminants caused by problems in the biological treatment and the final secondary decantation at the WWTP.

Table 2. Doses and contact times of the disinfectants.

	Chlorine dioxide	**Peracetic acid**	**Ultraviolet radiation**
Ring filter	8 mg/L T: 40 min	9.5 mg/L T: 11 min	59 mW·s/cm^2
Physico-chemical	5 mg/L T: 85 min	8.5 mg/L T: 11 min	94 mW·s/cm^2
Infiltration-percolation	5 mg/L T: 55 min	8.5 mg/L T: 11 min	97 mW·s/cm^2

T: contact time

Table 3. Characterization of the secondary effluent.

Parameter	Min.	Av.	Max.	St. Dev.
pH	7.8	8.4	9.4	0.5
Temperature ºC	15.5	21.5	27.9	3.5
Electrical conductivity (µS/cm)	1221	2076	2884	260
Dissolved oxygen (mg/L)	0.14	2.61	6.7	1.31
COD (mg/L)	17.0	69.9	242	46.2
Suspended solids (SS) (mg/L)	5.5	95.9	572	127.8
Turbidity (NTU)	3.0	42.8	267	54.5
Ca soluble (mg/L)	96.7	128.4	145.8	15.7
Mg soluble (mg/L)	24.6	33.7	38.2	4.3
Na soluble (mg/L)	169.0	246.2	304.7	46.8
K soluble (mg/L)	14.1	16.0	17.7	1.1
N-TKN soluble (mg/L)	4.3	8.8	13.9	3.7
$N\text{-}NH_4^+$ (mg/L)	<0.25	0.9	5.33	1.6
P soluble (mg/L)	<0.50	1.2	2.79	0.80
E. coli (Log cfu/100mL)	4.5	5	5.4	0.3
Total coliforms (Log cfu/100mL)	5.4	5.9	6.4	0.3
Somatic bacteriophages (Log pfu/100mL)	1.8	2.6	3.3	0.5

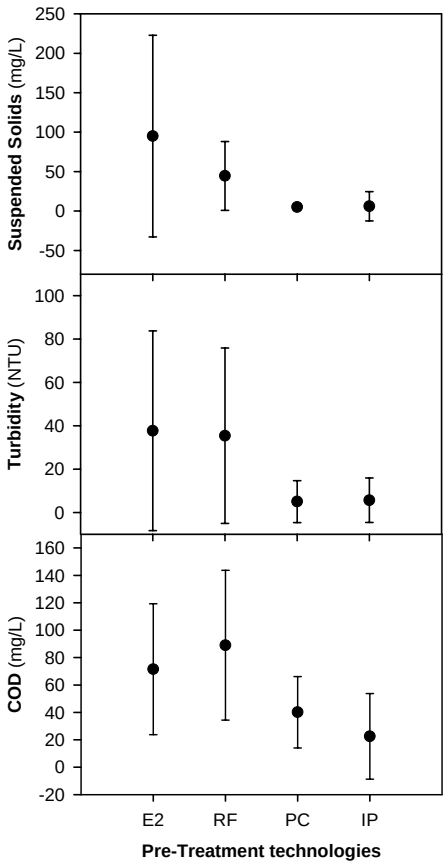

Figure 7. Average concentration and standard deviation of physicochemical parameters in the secondary effluent and the outlet effluent of each pre-treatment system.

The best removal rates of COD, SS, and turbidity are obtained by the infiltration-percolation system; this result can be explained based on its granulometric characteristics. (98% of particles are <1 mm in diameter) that determine the efficiency of the retention of suspended solids in the surface, and colloidal and dissolved solids in in the deepest area of the sand filter bed. It is to note that the sand filter depth (1.50 m) lengthens the filtration time, which should result in a better contaminant removal rate [12]. A substantial part of the organic matter is degraded by aerobic microorganisms which are part of the filter bed associated biofilm [13]. The efficiency of this infiltration-percolation system is comparable to the ones of infiltration-percolation systems with surface irrigation [14].

With respect to the physical-chemical system, SS reductions were very similar to those obtained with the infiltration-percolation system. The addition of chemicals in the physical-chemical system resulted in the aggregation of colloidal particles to form flocs that were subsequently decanted [15]. The lowest removal rates were obtained with the ring filter system, because only particles larger than the pore size of 25 μm were removed from the effluent. However, it should be noted that despite these apparently poor results, the ring filter system met the design specifications and would thus be completely efficient and adequate for less restricted wastewater uses. The reduction obtained in turbidity could be explained in the same way as the reduction in suspended solids, since both parameters are usually related.

The average concentrations and standard deviations for E. coli, total coliforms and somatic bacteriophages in the secondary effluent (inlet of each pretreatment system) and outlet effluent of the pretreatment technologies are presented in Figure 8. With regard to the mechanisms associated with the reduction in microorganisms yielded by the different technologies used, it should be borne in mind that some microorganisms were associated with solid particles and were therefore retained by the filtering mechanisms [15,16]. The infiltration-percolation system gave the highest removal rate, with an average E. coli reduction of 2.2 log cfu/100 mL. The physical-chemical and ring filter systems produced similar average reductions of 0.3 log cfu/100 mL, although the physical-chemical system achieved a slightly higher removal in some cases.

The removal of total coliforms was very similar to that observed for E. coli. The results show that the content of total coliforms was generally 2 log units higher than E. coli content, indicating that an important percentage of total coliforms was environmental rather than fecal in origin.

The mechanisms of disinfection in the infiltration-percolation system are associated with filtration, adsorption and predation. Filtration and adsorption are based on physical processes that achieve immobilization of pathogens

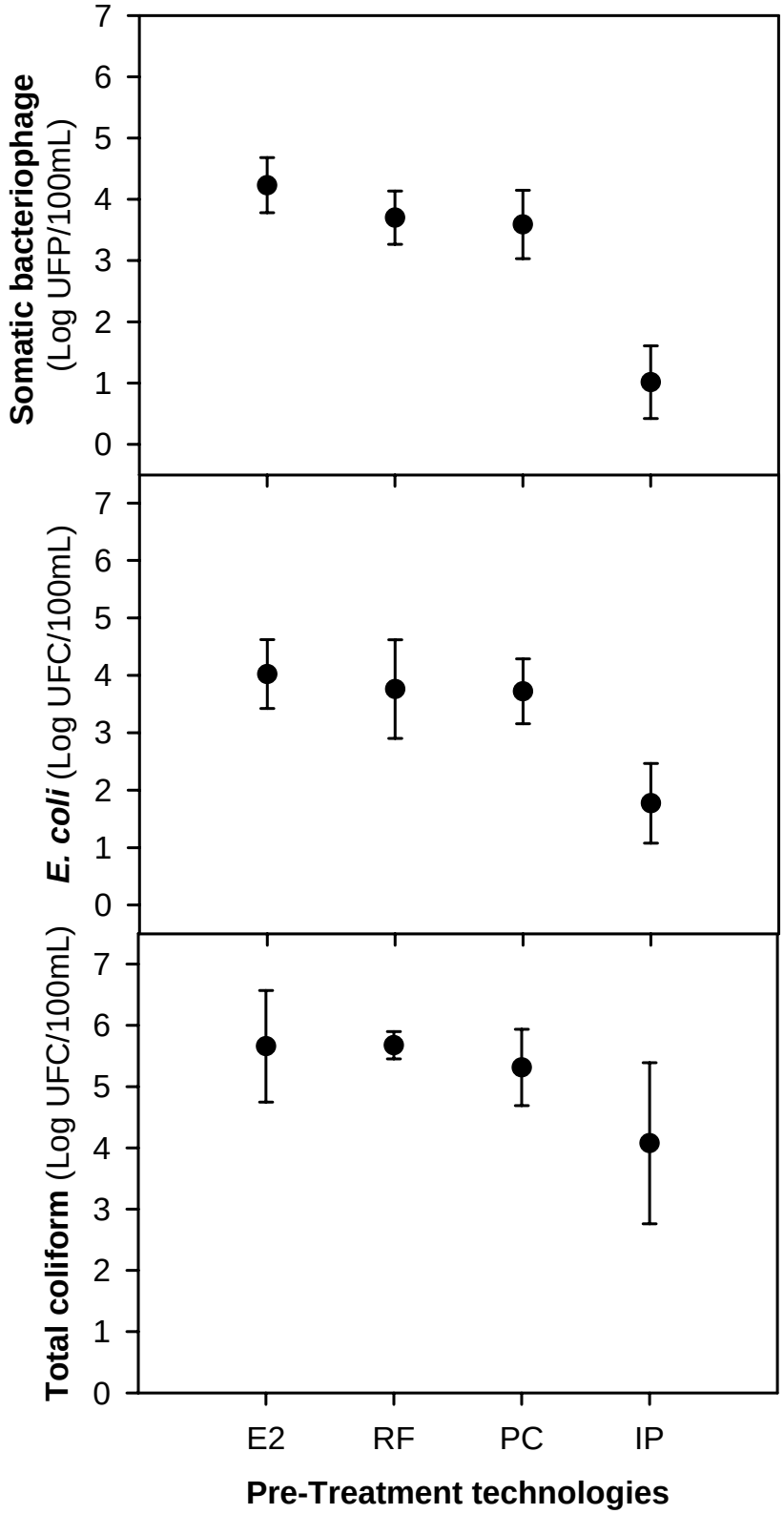

Figure 8. Average concentration and standard deviation of microbiological parameters in the secondary effluent and the outlet effluent of each pre-treatment system.

that are either associated with particles or free, while predation is a biological mechanism of competition typical of systems using biomass [17]. The system's hydraulic retention time plays a key role in the reduction of microorganisms.

The physical-chemical system reduced the concentration of bacteria because the microorganisms were trapped in the flocs formed once the chemical reagent had been added [18].

The infiltration-percolation system achieved the highest removal of somatic bacteriophages, with an average reduction of 3.2 log pfu/100mL, followed by the physical-chemical system (0.6 log pfu/100mL) and the ring filter system (0.5 log pfu/100mL). The virus removal mechanisms of each system were the same as those described for bacteria, although in some circumstances (heavy rains) bacteriophages could be desorbed [19, 20, 21].

2.1. Evaluation of the final reclaimed effluents quality

The results for microbiological water quality at the end of the treatment lines, following application of the disinfection technologies at the doses and contact times previously described, are shown in Table 4.

The treatment lines which achieved the highest removal of indicator microorganisms were those that used physical-chemical (PC) and infiltration percolation (IP) systems as pretreatments. The lowest removal rates were obtained for the RF+UV treatment line. This was an expected result, given that the high concentration of suspended solids due to the poor removal achieved by the ring filter interfered with the disinfectant action of the ultraviolet radiation, reducing its effectiveness [22, 23]. There were no significant differences between the disinfection efficiency of chlorine dioxide and peracetic acid, the most effective disinfectants evaluated, and the results are in agreement with other studies using similar doses and contact times [24,25].

According to the limits established by RD 1620/2007 for E. coli content in final effluents, the reclaimed water from the treatment lines employing physical-chemical and infiltration-percolation systems could be used for the urban, agricultural, industrial and recreational uses established in the Spanish legislation. The lines that used the ring filter system also met the requirements for the above-mentioned uses, except for urban uses, the most restrictive ones.

Table 4. Content and removal of microorganisms at the end of the treatment lines evaluated.

Treatment line	*E. coli* (log cfu/100mL)		Total coliforms (log cfu/100mL)		Somatic bacteriophages (log pfu/100mL)	
	Conc.	Rem.	Conc.	Rem.	Conc.	Rem.
RF+ClO_2	0.2	4.8	1.3	4.6	0.2	2.4
RF+PA	1.0	4.0	2.5	3.4	0.5	2.1
RF+UV	2.3	2.7	2.5	3.8	0.3	2.3
PC+ClO_2	0.4	4.6	1.2	4.7	0.0	2.6
PC+PA	0.3	4.7	1.2	4.7	0.3	2.3
PC+UV	0.9	4.1	1.9	4.0	0.0*	2.6
IP+ClO_2	0.4	4.6	1.1	4.8	0.0*	2.6
IP+PA	0.2	4.8	1.2	4.7	0.0*	2.6
IP+UV	0.6	4.4	0.5	5.4	0.0*	2.6

*: below detection limit (1 pfu/100mL); Conc.: final concentration; Rem: final removal.

3. Conclusion

The infiltration-percolation and physical-chemical pretreatment systems evaluated in this study showed consistent performance, being capable of homogenizing effluent quality during peak loads. Furthermore, infiltration-percolation is a "green" technology that is environmentally friendly since it does not require chemical additives for operation.

As regards wastewater disinfection, the chlorine dioxide and peracetic acid disinfection systems proved to be the most effective at the doses and contact times tested. The efficiency of ultraviolet radiation as a disinfectant was influenced by the presence of suspended solids which, as expected, decreased its effectiveness.

However, determination of the costs involved in the treatment line selected for a reclamation facility can be an important factor, and the ring filter may be more cost efficient for certain uses.

4. Future perspectives of the wastewater reclamation and reuse

Wastewater reuse remains the subject of research, and some of the leading researchers in the field continue to assess the hazards and health risks associated with wastewater reuse [26,27].

Drinking Water Safety Plans [28] have been implemented worldwide over the last three years, but nothing has been done in relation to the treatment, reclamation, distribution and reuse of wastewaters [29]. There is thus a need to design research methodologies and tools capable of generating the data and results necessary for correct evaluation and use of

reclaimed water with an acceptable risk, as established in the published recommendations.

The objectives to be achieved in the coming years, given the difficulties in adapting the Spanish RD 1620/2007 for wastewater reuse, include:

Implementation of the Hazard Analysis and Critical Control Points (HACCP) protocols, including determination of CCPs (Critical Control Points), calculation of DALYs (Disability Adjusted Life Years), calculation of QMRA (Quantitative Microbial Risk Assessment) and QCRA (Quantitative Chemical Risk Assessment), determination of acceptable risk, identification of risk reduction methods (using barriers, better technologies, etc.) and validation of methodologies.

Management of storage, distribution and application of reuse systems.

Monitoring of the environmental matrices receiving reclaimed water directly or indirectly (including protected areas).

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11. The effect of recombination in *Aeromonas*

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Abstract. Although several approaches have been attempted, the estimation of recombination frequencies in natural populations of bacteria remains challenging. Previous studies have demonstrated a wide variety of situations among bacterial species, ranging from the clonal diversification of *Salmonella* or *Escherichia coli*, which are mainly due to mutation, to the frequent recombination found in *Neisseria gonorrhoeae* or *Helicobacter pylori*. Most of the population studies done with bacterial species suggest that recombination occurs in nature but that it is infrequent compared to mutation. Consequently, bacterial populations consist largely of independent clonal lineages. Our research suggests little or null influence of recombination in the genetic structure of '*Aeromonas hydrophila* Species Complex', despite the presence of some strains with recombinant gene fragments.

Introduction

Bacteria reproduce asexually, giving two identical individuals after their division, with the exception of changes produced by mutation or recombination. Although this reproduction process is not associated with

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recombination, in contrast with eukaryotes, bacteria have acquired three basic mechanisms by which they can incorporate genes from other bacterial species: transformation, conjugation, and transduction. Nevertheless, although bacteria might incorporate foreign genes from other species, their genomes are not simply arbitrary assortments of genes of mixed heritage. Once the bacterial cells acquire genes by means of one of these mechanisms, they have to be incorporated into a replicon; if not, the introduced genes would become diluted in the population or degraded by the restriction endonucleases. Bacterial interchange promotes the acquisition of novel genetic elements, the impact of which has been extensively studied in human and animal pathogens and commensals, where they are often associated with the emergence of new phenotypes [1]. Recombination in bacteria is: always restricted to small DNA fragments, unidirectional, independent of reproduction, and occurs with a relatively low frequency, although genes codifying virulence factors or antibiotic resistance experiment more frequent recombination changes. The incorporation of genes or parts of genes through recombination always results in mosaic genomes that are composed of regions with different evolutionary histories [2]. Homologous recombination is widespread in the genomes of many bacteria, and is usually a consequence of *recA* mediated homology-dependant recombination. When the incorporated fragment replaces an identical DNA sequence its effect cannot be detected, although the process seems to be very frequent when the two bacteria involved in this interchange are closely related. Homologous recombination in this case might play a crucial role in DNA repair [3]. On the contrary, if recombination has a measurable effect on the genome of the recipient, it is considered as an effective recombination event.

The impact of recombination on bacterial phylogenies has been the subject of considerable discussion [4-9]. Recently, with the availability of sequencing techniques and the analytical power of new programmes, the detection of recombination events has increased dramatically. This has led to the questioning of existing phylogenies and the methods used for their construction, such as Maximum Likelihood (ML) and Maximum Parsimony (MP), which assume that the analyzed sequences have the same evolutionary history. The presence of recombination would break this assumption, since in this case sequences would have different underlying phylogenies that are more easily envisaged as a network rather than a tree. Due to the importance of recombination in evolutionary analysis, it is essential to be able to identify whether a given set of sequences has undergone recombination events, define the boundaries of the recombinational units, and evaluate the impact of recombination on our ability to reconstruct evolutionary histories and estimate population genetic parameters.

To investigate genetic exchange in bacteria, large data sources have been used, such as those deposited in the Multi Locus Sequence Typing (MLST) databases at www.mlst.net [10]. Multilocus data allow us to determine the degree of recombination based on the type of population structure. If the population shows a clonal structure (linkage disequilibrium), then recombination is absent and the accumulated genetic changes will be a consequence of mutation. However, in the case of bacteria, this assumption is not always true and the clonal structure is not always broken, even if a certain degree of recombination is present [11, 12].

The increasing availability of whole genome sequences has enabled a more complete study of the recombination process in bacteria. The genes sequenced in a MLST study (usually six or seven) are not always representative of the entire genome, and can give biased results. The analyzed sequences do not correspond to the complete gene, only fragments of about 400-500 bp, so the changes determined cannot be representative of the full gene, particularly in the case of a protein-codifying gene. Unfortunately, full genome sequences that could obviate all these questions are still usually limited to a few isolates of each species and frequently have been chosen for specific reasons (clinical, environmental or industrial, etc.), so are not representative of the entire population.

In this study we will consider the impact of recombination on bacterial phylogenies and the consequences of inaccurate approaches to inferring phylogenetic relationships. Traditionally, recombination in a given set of sequences has been identified by the incongruence of the different gene trees analyzed, the presence of mosaic structures, and variations in the G+C content or the codon bias. Several methods have been developed to test the presence of recombination in a given set of sequences, and to identify the parental and recombinant individuals or the recombinational break-points. Those methods can be classified in different categories: similarity, distance, phylogenetic, compatibility, and nucleotide substitution distribution [13, 14]. The performance of these methods varies depending on the level of recombination, but in general most of them are efficient, and although they can have trouble in detecting recombination when the level of divergence is low, their discriminatory power increases when the level of recombination is high [5]. In addition, methods that use the substitution patterns or incompatibility among sites seem to be more powerful than those based on phylogenetic incongruence. This might be partially explained by the fact that, in general, phylogenetic methods can only detect recombination events that change the topology of the tree, and at high recombination rates there should be many such events [5]. What is important is to increase the chances of detecting recombination while minimizing the detection of false positives, so

the chosen method will depend on the level of divergence among the sequences analysed [15, 16]. In either case, the best option is not to rely on a single method to detect recombination [13, 17].

1. Recombination in bacteria

Although several approaches have been attempted, the estimation of recombination frequencies in natural populations of bacteria remains challenging. One of the parameters commonly used is the determination of the rate of recombination relative to mutation [18], but this is not always easy to calculate, except in the case of the most recent events [4], which might not be representative of the entire history of the population.

The determination of the relative importance of mutation in comparison with recombination is central to bacterial population genetics [18]. Previous studies have demonstrated a wide variety of situations among bacterial species, ranging from the clonal diversification of *Salmonella* [19] or *Escherichia coli* [20], which are mainly due to mutation, to the frequent recombination found in *Neisseria gonorrhoeae* [21] or *Helicobacter pylori* [22]. Most of the population studies done with bacterial species suggest that recombination occurs in nature, and indeed may be highly important in generating variation, but that it is infrequent compared to mutation. Consequently, bacterial populations consist largely of independent clonal lineages.

Comparison of results from analyses performed with different methodologies is problematic; nevertheless, studies using the same methods across different genera have suggested wide variation in recombination rates with value differences of two or three orders of magnitude [23]. In addition, conflicting levels of recombination have been obtained for concrete bacterial species, such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus* or *Haemophilus influenzae* [24], depending on the sampling and analytical methodologies used. The analyzed isolates have to be sampled carefully in order to be informative about the underlying recombination process. If they are not representative of the whole population, an important bias might be introduced. Unfortunately, in most of the populations studied, particularly in the case of pathogen bacteria, samples are not fully representative, usually with an overrepresentation of virulent strains, which are frequently under higher selective forces than the corresponding non-virulent strains.

Recombination studies using whole genome data have contributed to a better understanding of recombination in bacteria. Several studies have reported differences in the prevalence of recombination at different regions of the same bacterial genome [25], being apparently higher in those genes under

positive selection [25, 26]. Genomic regions encoding proteins with a role in pathogenicity are often under positive selection and frequently exhibit high rates of recombination, even in the case of bacteria in which recombination is relatively rare [28, 29]. A possible explanation for the relative prevalence of recombination in positive selected regions of the bacterial genome is that the only observable recombination is likely to be the one that unites beneficial mutations and removes deleterious ones.

2. The genus *Aeromonas*

The genus *Aeromonas* Stanier 1943 belongs to the family *Aeromonadaceae* within the class *Gammaproteobacteria* [30]. Aeromonads are autochthonous inhabitants of aquatic environments, including chlorinated and polluted waters, although they can also be isolated from a wide variety of environmental and clinical sources. They cause infections in vertebrates and invertebrates, such as frogs, birds, various fish species, and domestic animals. In recent years, some authors have considered *Aeromonas* as an emergent pathogen in humans, producing intestinal and extraintestinal diseases. Aeromonads are facultative anaerobic chemoorganotrophs capable of anaerobic nitrate respiration and dissimilatory metal reduction [30].

Several attempts have been made to generate phylogenies using DNA gene sequences to reconstruct the correct genealogical ties among species in *Aeromonas* [31-33], but the genes chosen for this purpose are not always suitable, and do not necessarily give congruent phylogenies [34, 35]. Recently, two papers presenting MLST schemes for *Aeromonas* have been published [10, 36], and there is an online MLST database for the genus *Aeromonas*, managed by Keith Jolley and curated by Barbara Cardazzo (http://pubmlst.org/aeromonas). All this accumulated data should help to establish a reliable clustering of the *Aeromonas* species and elucidate their exact boundaries.

Finally, the availability of complete genomes of different species is also useful in this task, but unfortunately, in the case of *Aeromonas*, only six genomes have been completed to date, corresponding to: the type strain of *A. hydrophila* ATCC 7966, isolated from a tin of milk [37]; the strain A449 of *A. salmonicida*, a fish pathogen described by Reith *et al.* ([38], Fig. 1); an *A. caviae* strain Ae398 isolate from a stool sample [39]; an *A. veronii* strain B565 isolated from an aquaculture pond sediment [40]; and more recently, an *A. aquariorum* strain AAK1 isolated from blood [41] and the highly melanin-yielding *A. media* strain WS [42]. The information given by the genomes of *A. hydrophila* and *A. salmonicida* indicates that while they are of identical size (4.7Mb) and share multiple housekeeping and virulence genes, *A. salmonicida*

has acquired several mobile genetic elements, and undergone genome rearrangements and loss of genes in the process of adapting to a specific host. The genome of *A. veronii* is smaller (4.3Mb) and contains fewer virulence genes. Similarly, *A. caviae* presents a small genome (4.43Mb), but in contrast to *A. veronii* several putative virulence genes have been identified, as in *A. aquariorum*, which has the biggest genome reported for *Aeromonas* (4.81Mb). The last genome completed corresponds to an *A. media* strain (4.3Mb) in which no virulence genes have been reported.

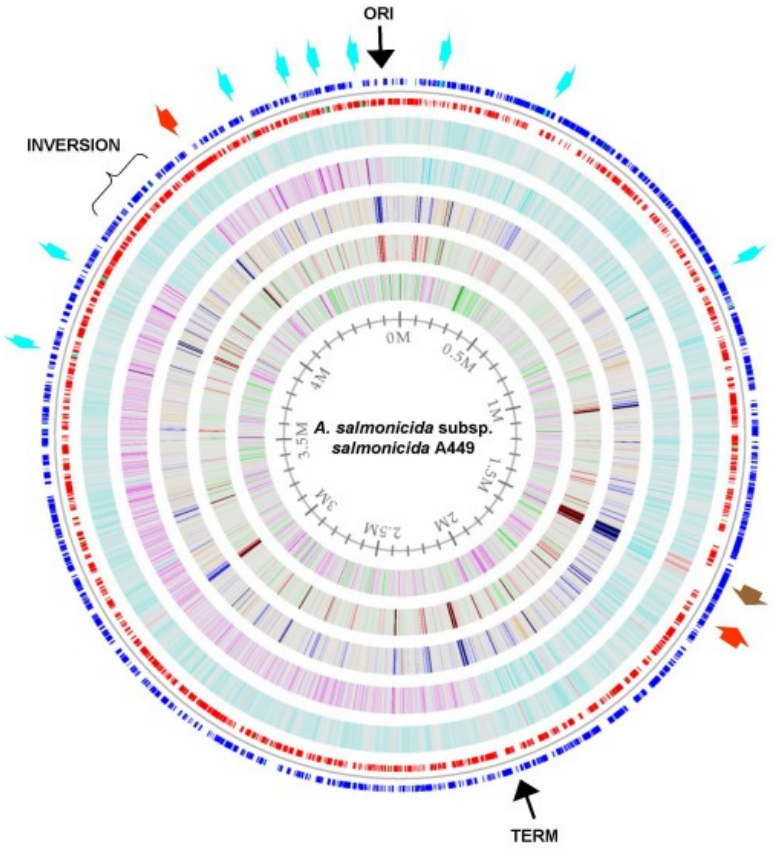

Figure 1. A genome atlas representation of the *A. salmonicida* subsp. *salmonicida* A449 chromosome (ref. [38]).

3. The '*Aeromonas hydrophila* species complex'

An example of the taxonomic complexity of the genus *Aeromonas* is the difficulty in discriminating between the phenotypically and genetically closely related species belonging to the "*Aeromonas hydrophila* species complex" (AHC), which includes: *A. hydrophila*, composed of three subspecies: *A. hydrophila* subsp. *hydrophila, A. hydrophila* subsp. *ranae* and *A. hydrophila* subsp. *dhakensis*, *A. bestiarum*, *A. popoffii*, and *A. salmonicida,*

divided in five subspecies: *A. salmonicida* subsp. *salmonicida*, *A. salmonicida* subsp. *masoucida*, *A. salmonicida* subsp. *achromogenes, A. salmonicida* subsp. *pectinolytica*, and *A. salmonicida* subsp. *smithia* [30, 43]. Recently, two additional species have been described in this group, *A. aquariorum* and *A. piscicola* [44, 45]. Members of the AHC were first described as strains producing the enzymes elastase, lecitinase or stapholysin [46]. They are genetically closely related and share multiple phenotypic characteristics, which makes discrimination among the species included in this group extremely difficult [43].

In order to establish the population structure and divergence of the species included in the AHC group, Fusté *et al.* [12] studied a set of representative strains, in which they analyzed the nucleotide sequences (total or partial) of 6 housekeeping genes. The authors concluded, from the linkage disequilibrium analysis and sequence divergence results, that the AHC is composed of four robust groups that basically correspond with the phenotypically described species *A. hydrophila*, *A. bestiarum*, *A. popoffii*, and *A. salmonicida*, in which recombination, if present, does not break their clonal structure.

4. Population structure and recombination in *Aeromonas*

The few references in the bibliography dealing with recombination in *Aeromonas* reach similar conclusions about its low incidence, with the exception of the study by Silver *et al.* [35], which reports a notable effect of recombination in the "*A. veronii* species complex". In this study, the strains were obtained from patients, veterinary samples, and medicinal leeches. The aligned sequences were investigated for evidence of recombination because the maximum-likelihood inferred phylogenies for each gene family showed low bootstrap support for most clades. Appling two tests for recombination, employing a variety of approaches, it was demonstrated that at least for some strains, horizontal gene transfer occurs at a sufficient frequency to blur the signal from vertically inherited genes, despite strains being adapted to distinct niches.

Martino *et al.* [11] analyzed a collection of *Aeromonas* including 23 type and reference strains, and 77 strains isolated from fish, crustaceous and molluscs in a MLST study using 6 housekeeping genes. Based on an eBURST analysis, the authors determined the recombination/mutation (*r/m*) ratio for the entire population and for the three major groups identified. The *r/m* values obtained (ranging between 0.07 and 0.13) suggest a reduced rate of recombination. Analysis with the SplitsTree program revealed that most of the genes, although showing a reticulate network, were not significantly affected by intragenic recombination with the exception of *recA*. When a set

of programmes included in the RDP3 package were applied to the *Aeromonas* sequences, several recombination events were identified supported by at least three of those programmes. Nevertheless, they concluded that, in the case of *Aeromonas*, the impact of recombination may be negligible, based on the very similar topologies of the phylogenetic trees, the low *r/m* rates, and the reduced network structure determined by the split decomposition analysis.

Roger *et al.* [47], in a MLST study that includes isolates from different origins but with a particularly high representation of clinical strains, determined that the standardized I_A (I_A^S) values showed the existence of significant linkage disequilibrium, indicative of a clonal population structure. When using at least four methods of the RDP software, they detected some recombination among the population in all but two of the seven loci analyzed. In addition, the use of split decomposition determined that most of the sequence types (STs) were not affected by recombination, even though more recombination events were found within the clonal complexes, particularly for the STs in the *A. caviae* clade. Differences in branching order were observed in both distance and ML trees when gene and concatenated sequence trees were compared, suggesting the occurrence of recombination. The authors conclude that recombination is present in the genus *Aeromonas*, at least in some strains, but at a relatively low frequency.

Finally, our group determined the genetic population structure of a group of *Aeromonas* corresponding to the AHC [12], which had been previously analyzed by enzyme electrophoresis (MLEE), revealing a clear clonal structure with strong linkage disequilibrium among 15 different protein loci [48]. We used a higher number of AHC isolates including representatives of *A. piscicola* [45] and *A. aquariorum* [44], two more recently described strains grouped within the AHC. The I_A^S values obtained in this study were different from 0 in all cases, indicating the absence of recombination and again, revealing strong linkage disequilibrium when considering both the total population and the different sets of species. This is in spite of the high number of alleles per locus and polymorphic sites and huge genetic diversity.

During the last years, with the availability of the first DNA sequence data of individual genes, evidence of recombination at the molecular level has accumulated for *Aeromonas* in housekeeping genes such as *dnaJ*, *gyrB* and *recA* [11, 35, 47] or structural and accessory genes [35]. In our study we have also determined the presence of potential recombinant fragments in the *recA* and *dnaJ* genes of some strains. However, although these strains cluster separately when the corresponding gene tree is constructed, revealing a possible different origin of the gene fragments, they group together with the other strains of the same species when a concatenated tree is generated. This confirms that recombination is not sufficient to break the genetic cohesion of this group.

5. Intragenic recombination in the *dnaJ* gene of the '*Aeromonas hydrophila* species complex'

We have recently studied the possible existence of recombination in the *dnaJ* gene (Fig. 2) in an AHC group with 90 strains (87% of environmental and 13% of clinical origin). Group I included 29 *A. salmonicida* strains (5 subspecies), Group II 31 strains (22 *A. bestiarum*, 5 *A. popoffii* and 4 *A. piscicola*) and Group III 30 strains (26 *A. hydrophila* and 4 *A. aquariorum*). Gene sequences used were obtained from our previous work [12]. We added a new strain *A. hydrophila* JCM 3968 (GenBank accession numbers: JN711671 (*dnaJ*), JN711562 (*cpn60*), JN711795 (*gyrB*), KC525968 (*mdh*), KC525969 (*recA*) and JN712375 (*rpoD*)). We determined the presence of mosaic structures, different G+C content and codon usage bias in the sequences but none of the results were conclusive.

In our study we also detected incongruences in the phylogenies when the *dnaJ* gene and the concatenated trees were compared (Fig. 3). Five strains, 2 *A. bestiarum* (orange), 1 *A. hydrophila* (blue), and 2 *A. salmonicida* (green), clustered out of the corresponding species group in the *dnaJ* gene tree (Fig. 3a), revealing a possible different origin of the gene fragments. Nevertheless, they grouped together with the other strains of the same species when a concatenated tree was generated (Figs. 3b and 4).

Consequently, we also analyzed our sequences using six recombination detection programmes in the RDP4 package, which significantly detected possible recombination events in one (*A. salmonicida* CECT 5214) out of the five strains, when all strains or the corresponding species subgroups were analyzed (Table 1). The investigation also provided well-supported evidence for recombination in two *A. hydrophila* strains (CECT 4330 and CECT 5734), which clustered among the other *A. hydrophila* isolates in the *dnaJ* and the concatenated trees, although they were separated in a deeper branch. No recombination events were detected among the *A. bestiarum* isolates (Group II).

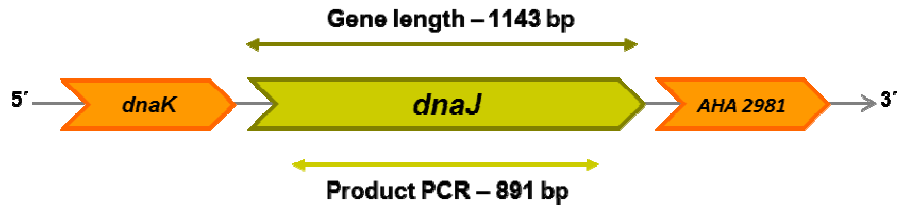

Figure 2. Schematic representation of the *dnaJ* locus and its flanking regions for *Aeromonas*, based on the whole genome sequence of *Aeromonas hydrophila* ATCC 7966^T (GenBank accession number CP000462, [37]). Partial sequences for *dnaJ* (891 bp) were obtained from the GenBank database or determined as previously described [49].

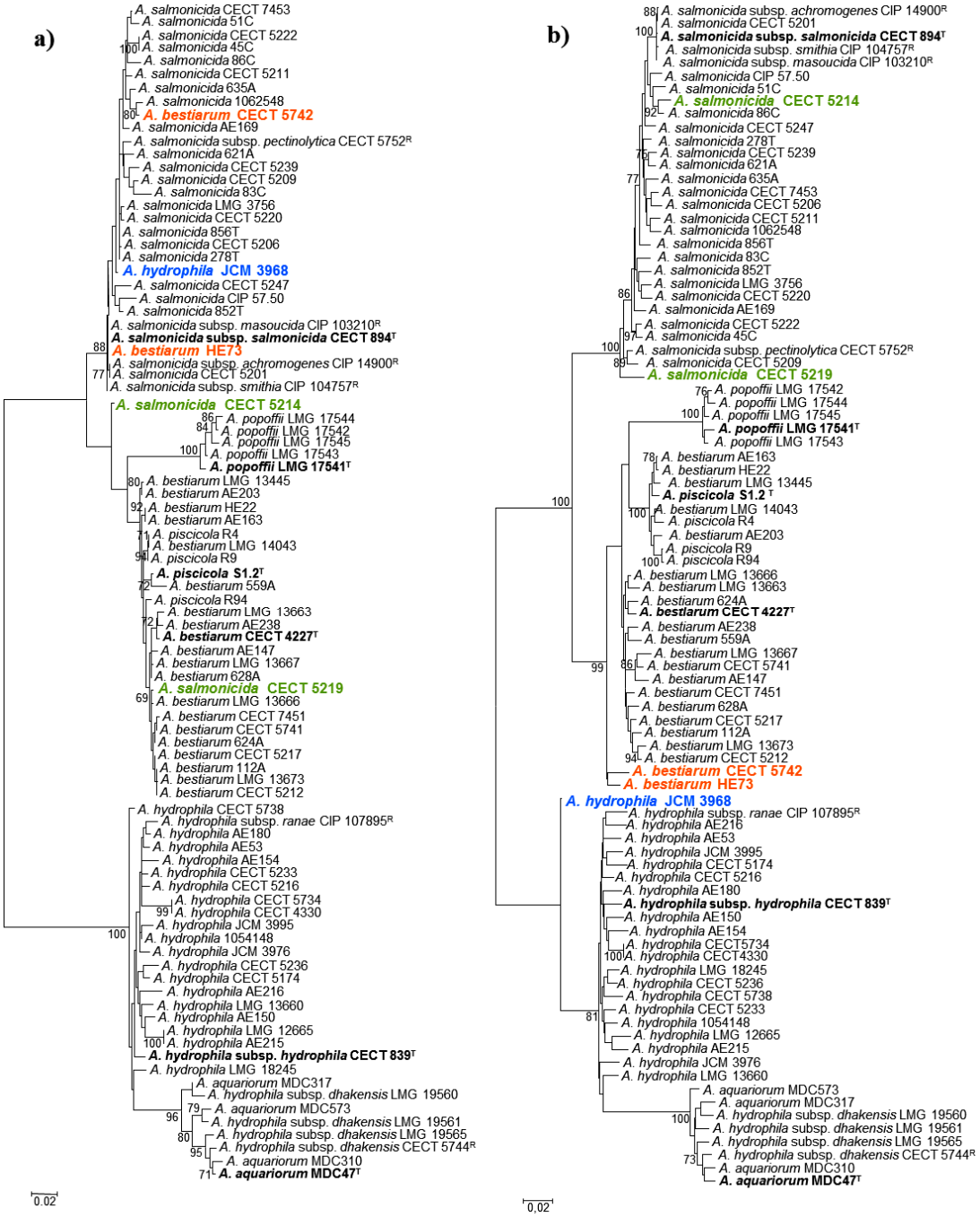

Figure 3. Phylogenies of AHC species inferred from single and concatenated genes: a) Maximum likelihood (ML) tree obtained from *dnaJ* sequences (891 positions) based on the Tamura-3-parameter (T92+G+I) as a model of nucleotide substitution; b) ML tree from concatenated sequences of six genes (5,379 positions) based on the Tamura-Nei model (TN93+G+I). ML trees were constructed using MEGA5 software (http://www.megasoftware.net, [50]). Bootstrap values (≥70%) from 500 replications are shown at the nodes of the tree. The scale bar indicates the number of nucleotide substitutions per site. The type strains of *Aeromonas* species belonging to AHC are indicated in bold. The five strains of the AHC in which we detected incongruences in the *dnaJ* tree are shown in colours.

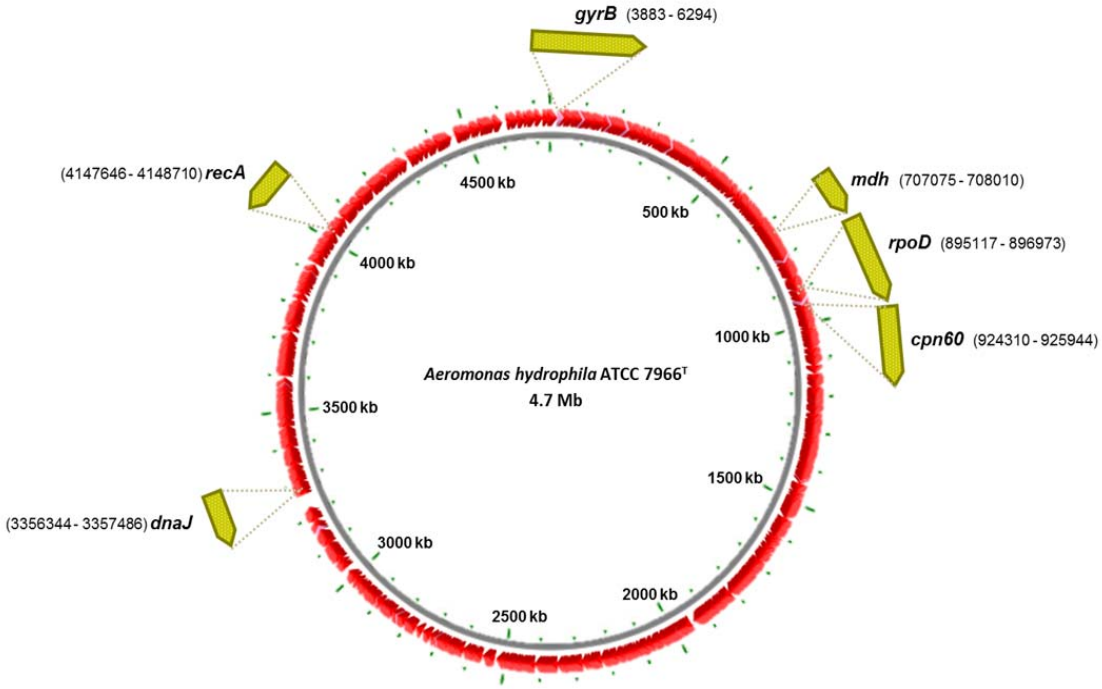

Figure 4. Distribution of six genes sequenced in the circular map of the genome of *A. hydrophila* ATCC 7966^T (GenBank accession number CP000462, [37]). Genes are shown outside the circle and have standard abbreviations. Arrows indicate direction of transcription. Detailed genomic position is listed in parentheses after each gene name.

Table 1. Recombination analysis summary.

	Recombination sequence	Number of methods detecting recombination events with statistical significance	Recombination detection methods						Events
			1	2	3	4	5	6	
***All strains* N=90**									
	A.salmonicida CECT 5214	3	■		■			■	1
Group I *A. salmonicida* **N=29**									
	A.salmonicida CECT 5214	4		■	■	■		■	1 or 2
	A.salmonicida CECT 5219	1				■			1
Group III *A. hydrophila* **N=30**									
	A.hydrophila CECT 4330	2				■		■	1
	A.hydrophila CECT 5734	2				■		■	1

Automated screening for recombination from multiple alignment of *dnaJ* sequences was performed using programme RDP4 (http://darwin.uvigo.es/rdp/rdp.html, [51]), which used six recombination detection programmes: RDP (1), GENECONV (2), BootScan (3), MaxChi (4), Chimaera (5) and 3Seq (6), with their default parameters. Sequences statistically supported by at least two recombination detection programmes (*P*-value <0.05) were considered as possible recombinants.

6. Conclusions

Developments in gene sequence analysis have greatly enhanced the study of recombination in bacterial populations. Gene-wide approaches to mapping bacterial diversity, which have already proved effective for gaining insight into bacterial evolution, have the potential to reveal the phenotypic basis of genetic diversity in *Aeromonas*, and to investigate the dynamics of this complex bacterial community. The objective of the work described in this chapter has been to evaluate the importance of the presence of recombination events and their influence on phylogenies, as it has been frequently postulated that in bacterial populations, horizontal gene transfer (HGT) is so common that it precludes the existence of biological species. Our research suggests little or null influence of recombination in the genetic structure of AHC species, despite the existence of some strains with recombinant gene fragments.

Assuming that the cohesion of major phylogenetic groups within the prokaryotes is due to vertical transmission and common ancestry rather than preferential HGT, it is possible to construct robust phylogenies reflecting the evolutionary history of bacteria, using a sufficient number of orthologous housekeeping genes (concatenated trees). In these phylogenies, bacterial species are delineable as 'classical Darwinian' evolutionary lineages [52-55].

The foregoing consideration does not exclude the existence of horizontal gene transfer, which in fact occurs, and has important evolutionary consequences, but it is doubtful that HGT is the essence of modern genome phylogeny [53]. Moreover, as demonstrated in *Salmonella, Streptococcus,* and *Bacillus*, homologous recombination decays exponentially with sequence divergence; in other words, a sequence divergence between two strains of 10% suppresses the recombination rate between them by a factor of about 100 [56, 57]. We are currently pursuing recombination studies in other genes of this *Aeromonas* group.

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12. Is leishmaniosis spreading to northern areas of the Iberian Peninsula? The examples of Lleida (NE Spain) and Andorra

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Abstract. The entomological and canine leishmaniosis surveys carried out in the northwest of Catalonia and in Andorra in the context of the European project Emerging Diseases in a changing European eNvironment (EDEN) are summarized. The aim of the study was to obtain data on the presence of leishmaniosis in these areas and the spatial distribution of their vectors.

Introduction

Global changes in climate, human activities and migration have resulted in the emergence or re-emergence of vector-borne diseases, such as leishmaniosis, in some parts of the world [1-5]. Leishmaniosis is a transmissible disease that affects man and other mammals and is caused by different species of the genus *Leishmania* [6]. In Europe it is caused by *Leishmania infantum* and dogs are the principal reservoirs [2].

The highly specialized transmission of leishmaniosis is by the bite of hematophagous insects acting as vectors and is restricted to a group of dipterans,

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the phlebotomine sand flies [2,7], which are found mainly in warm parts of the world [8]. Two genera are involved in the transmission of leishmania parasites: *Phlebotomus* in the Old World and *Lutzomyia* in the New World. In Spain, two species of the genus *Phlebotomus*, subgenus *Larroussius*, *P. ariasi* and *P. perniciosus*, are the proven vectors, acting in sympatric conditions in some of the foci [3,9-11]. Studies carried out on phlebotomine richness in Spain identified 12 sand flies species, 10 of the *Phlebotomus* genus and 2 of *Sergentomyia*, each one with a different distribution in the Iberian Peninsula [12-17]. As in other countries, the focalized geographical distribution of sand flies in Spain has been related with climatic and environmental characteristics, but few in-depth studies exist on the influence of such variables on the distribution of sand flies in local areas [17-19].

Human leishmaniosis (HL) is present in 98 countries around the world [20]. In humans, the disease is usually manifested as visceral or tegumentary forms, cutaneous cases being more frequent [4]. In recent years, the cryptic form of HL has also been described, mostly among blood donors [21]. Until recently, in Europe HL was considered to be present mainly in the Mediterranean Basin, but it is now spreading and emerging in zones previously thought to be free, as well as increasing in endemic areas [22-26]. HL has been reported in northern and eastern countries of Europe, although some cases are considered imported [27]. In Spain, the first case of HL was described by Pittaluga in 1912 [28], after which many cases were diagnosed in the Iberian Peninsula. The disease began to decrease at the end of the 1940s, probably due to the reduction of vectors by insecticide use and also a decline in reservoirs after a civil war and a period of severe economic depression [11,29,30]. In the 1980s the disease increased considerably due to opportunistic infection in VIH patients [31]. From 1982 until 1996, HL was a notifiable disease in Spain, but its notification is currently mandatory in only 12 of the country's 17 autonomous communities, including Catalonia.

Canine leishmaniosis (CanL) is a widespread disease, present in 42 countries in Europe, Africa, Asia and America, and suspected in three other countries of Africa [2]. The disease is included in the list of the World Organization for Animal Health (OIE), being considered as important from the socio-economical and sanitary points of view. As dogs constitute the main reservoir hosts of the parasite, this represents a risk for humans [2,3], particularly considering the high number of asymptomatic animals [32-34]. In Western Europe, CanL is considered highly endemic in some parts of Portugal, Spain, France and Italy. In recent years, the disease has been increasing in classical areas [19,25,26] and has spread to northern regions considered non-classical for CanL [23,24,35]. However, cases in northern and eastern Europe have been detected in dogs that have travelled with their

owners to the Mediterranean basin or been bought in endemic areas [27]. CanL can manifest itself with a great variety of unspecific cutaneous and visceral symptoms, which hampers clinical diagnosis [36]. Cryptic leishmaniosis in dogs has been described [36,37]. The first case of CanL in Spain was reported by Pittaluga in 1913 [38]. Although nowadays the disease is very widespread in the country and well known by veterinarians, no official data exists on its prevalence, which seroepidemiological studies situate in a range of 1.6% in the North to 34.6% in the South [39,40]. The data on CanL distribution is also incomplete, with very little available for the north of the country.

The present work summarizes epidemiological studies on leishmaniosis carried out in Andorra and Spain (Catalonia, Lleida province) in the course of the European project Emerging Diseases in a changing European eNvironment (EDEN).

1. Andorra. Entomological survey

Andorra is a small European country of 468 km^2 located in the Pyrenees, consisting predominantly of rugged mountains, with the lowest point at 840 m a.s.l., in the southern basin of the Gran Valira, and the highest at the 2946 m Coma Pedrosa peak. It is characterized by a Mediterranean high mountain climate with cold temperatures in winter and mild summers, with considerable variations depending on altitude and orientation. The country is divided in 7 administrative parishes: Canillo, Encamp, Escaldes-Engordany, La Massana, Ordino, Sant Julià de Lòria and Andorra La Vella.

No data exist on leishmaniosis in Andorra, despite the disease being well known in its neighbouring countries of Spain and France [24,37,41]. The only existing report on the phlebotomine fauna in this country describes the survey of one collection site, where two sand fly species were captured, *S. minuta* and *P. ariasi* [15]. Since the presence of the vector is the focusing element for a transmissible disease and data on sand fly vectors in Andorra was lacking, we decided to carry out an entomological survey.

39 stations were sampled during July 2007 covering all the parishes except Andorra La Vella. Sticky traps (20 cm× 20 cm sheets of papers covered in castor oil) were used and set in adult sand fly resting places [7,42] for four days (Fig. 1). Because of the highly mountainous topography of the region, captures were made along transects [7], following all the main roads. The sand flies were fixed until their morphological identification following the keys of Gállego Berenguer et al. (1992) [13].

Figure 1. Sticky traps used in the capture of sand flies: Examples of a sampling site and adhesive paper with captured sand flies.

A total of 21 specimens belonging to two species of the genus *Phlebotomus*, *P. ariasi* (5 females, 13 males) and *P. perniciosus* (1 female, 2 males) were captured in 10 sampling sites [43]. Global results of number, density, abundance and frequency are summarized in Fig. 2. The characteristics of vector density according to altitudinal distribution are summarized in Fig. 3.

The geographical distribution of the vectors and their densities are represented in Fig. 4. *P. perniciosus* is restricted to the south of the country, in Sant Julià de Lòria parish, at altitudes below 1,000 m a.s.l., whilst *P. Ariasi* covers a wide area, from 800 m to 2,200 m a.s.l., being present in all the 6 parishes where traps were placed [43]. The results obtained in Andorra are in

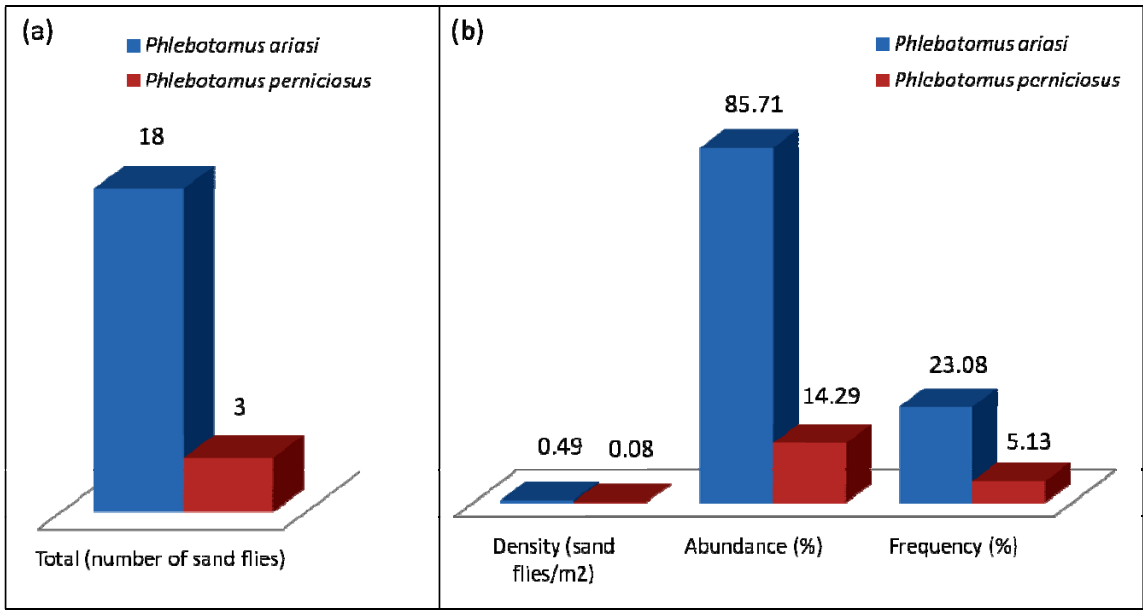

Figure 2. (a) Number, (b) density (number of sand flies per square metre of sticky trap), abundance (relative number of captured sand flies of one species related to the total number of sand flies captured and expressed as a percentage) and frequency (relative number of positive stations for one species expressed as a percentage) of *P. ariasi* and *P. perniciosus* in Andorra.

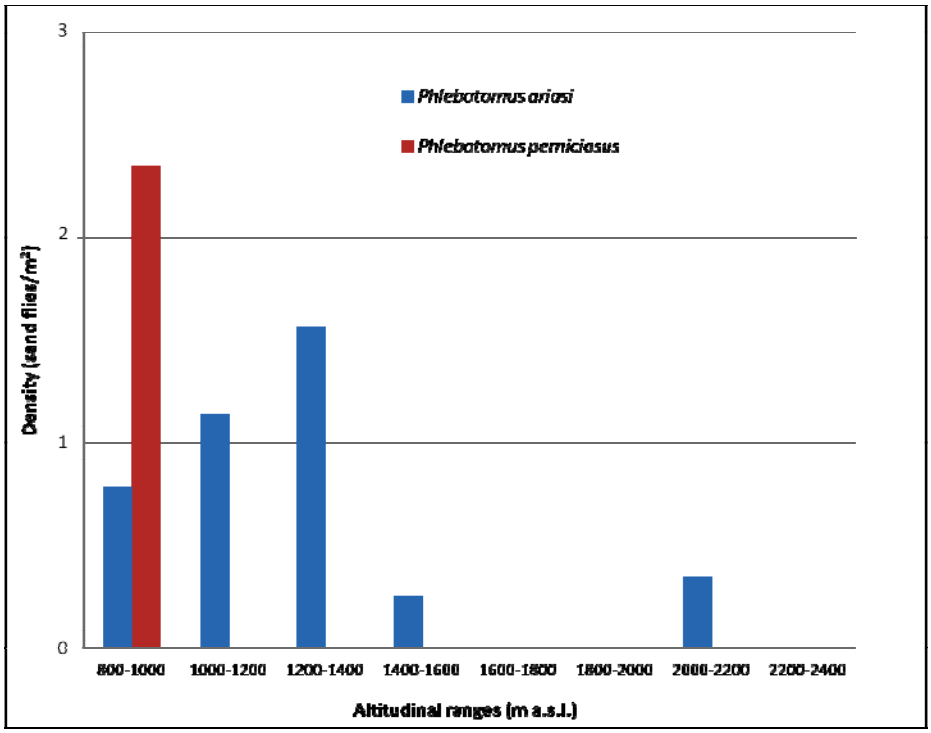

Figure 3. Density (number of sand flies per square metre of sticky trap) of *P. perniciosus* and *P. ariasi* at different altitudinal ranges in Andorra.

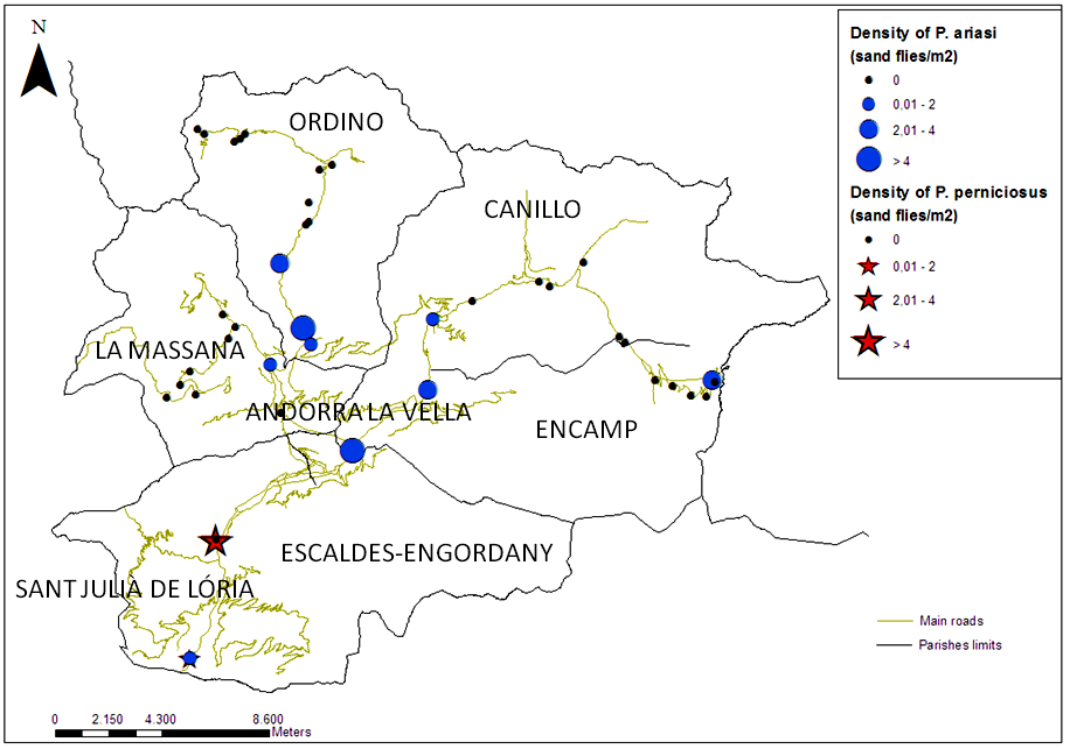

Figure 4. Sampling sites in Andorra showing the presence and density ranges of *P. ariasi* and *P. perniciosus*.

agreement with the predominant presence of *P. ariasi* in humid or sub-humid areas with cold winters, being the only potential vector of leishmaniosis in cold zones, and the preference of *P. perniciosus* for semi-arid or subhumid zones with warm winters and mild summers [44,45].

This entomological survey resulted in the first reporting of *P. perniciosus* in Andorra, and gave new insight into the geographical and altitudinal distribution of *P. ariasi* in this country. *P. ariasi* was captured at altitudes above 2,000 m a.s.l., which is unusual in the Mediterranean area [46].

Vector presence is considered to be a risk factor for the emergence of leishmaniosis in temperate Europe [3]. As both species are responsible for the transmission of *L. infantum* in bordering France and Spain [24,44], we therefore consider Andorra to be an area at risk for the emergence of the disease. The arrival of infected dogs from endemic areas, without surveillance and control measures, could lead to the establishment of the disease in this country [43]. The summer temperature in Andorra would allow the development of the leishmania life cycle in the vector, as has been experimentally demonstrated for *P. ariasi* [7], and its transmission to the vertebrate hosts.

2. Lleida province

The province of Lleida is situated in the NE of Spain (Catalonia), bordering with the Pyrenean areas of Andorra and France in the North. It covers an extension of 12,173km^2 and occupies a great variety of habitats, including plains and mountains (Pyrenees). The altitude ranges from just over sea level to 3,143 m a.s.l. of the Pica d'Estats mountain. Climate is highly variable according to altitude, ranging from Mediterranean High Mountain in the mountainous areas in the north to a continental climate in the central depression.

Before our survey, only one partial entomological survey had been carried out in Lleida province [44], reporting a sand fly fauna of 5 species (*S. minuta*, *P. perniciosus*, *P. ariasi*, *P. sergenti* and *P. papatasi*), including the vectors of *L. infantum*. Some HL cases had been noted in the epidemiological bulletins [47], but no data on CanL was available.

Due to the limited knowledge of sand fly fauna in Lleida, we decided to carry out an entomological study throughout the province. The EDEN methodology was applied so the results would be comparable with those obtained by our survey in Andorra and by other European teams participating in the project. Another aim was to obtain HL and CanL data in the province for the first time.

2.1. Entomological survey

A cross-sectional study was carried out in July 2006 in Lleida province. Sand flies were captured using sticky traps, as in Andorra. A total of 4100 sticky traps were placed in all the 13 counties of the province. Due to the large extension and physical characteristics of the region, captures were made by transects following the main roads, which enabled the whole territory to be covered [7].

Five species of sand flies were captured, the densities shown in Table 1. All were previously identified in the province [44]. The results obtained show that the two vector species of *L. infantum* are present in Lleida province. Neither species was found in Pla d'Urgell county. *P. ariasi* was not present in the southern counties (Garrigues and Segrià), probably because this species mainly inhabits humid or sub-humid zones with cold winters, and *P. perniciosus* was not found in Vall d'Aran, which is the coldest and wettest county.

Table 1. Density (number of sand fly species captured per square metre of sticky trap).

Density (sandflies/m^2 sticky trap)					
County	*P. ariasi*	*P. perniciosus*	*P. sergenti*	*P. papatasi*	*S. minuta*
Alt Urgell	1.47	2.76	0	0	33.41
Alta Ribagorça	2.74	3.48	0	0	13.37
Garrigues	0	6.56	0	2.38	31.62
La Cerdanya	0.31	1.25	0	0	0.94
Noguera	0.49	12.86	0.22	0.45	33.04
Pallars Jussà	1.78	4.83	0.77	0.05	76.14
Pallars Sobirà	1.70	1.51	0	0	54.91
Pla d'Urgell	0	0	0	0	77.68
Segarra	0.06	7.84	0.48	3.13	22.96
Segrià	0	0.91	0	0.86	16.77
Solsonès	2.09	1.83	0	0.04	1.44
Urgell	0.03	7.85	0.07	1.29	22.33
Vall d'Aran	0.98	0	0	0	0

Global results on the number, density, abundance and frequency of leishmania vectors are summarized in Fig. 5. The characteristics of vector density according to altitudinal distribution are summarized in Fig. 6 and their geographical distribution among the counties of Lleida is included in Fig 7.

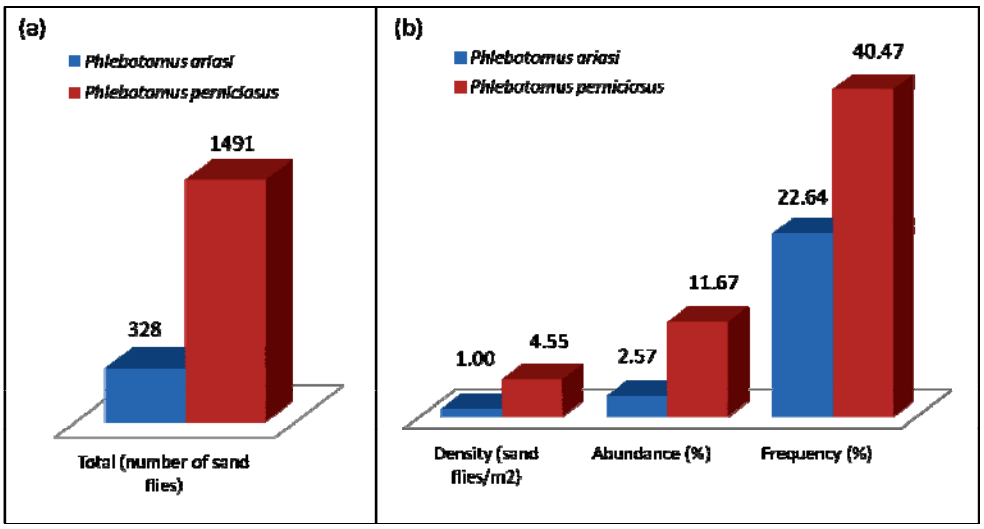

Figure 5. (a) Number, (b) density (number of sand flies per square metre of sticky trap), abundance (relative number of sand flies of one specie captured related to the total number of sand flies captured and expressed as a percentage) and frequency (relative number of positive stations for one specie expressed as a percentage) of *P. ariasi* and *P. perniciosus* in Lleida province.

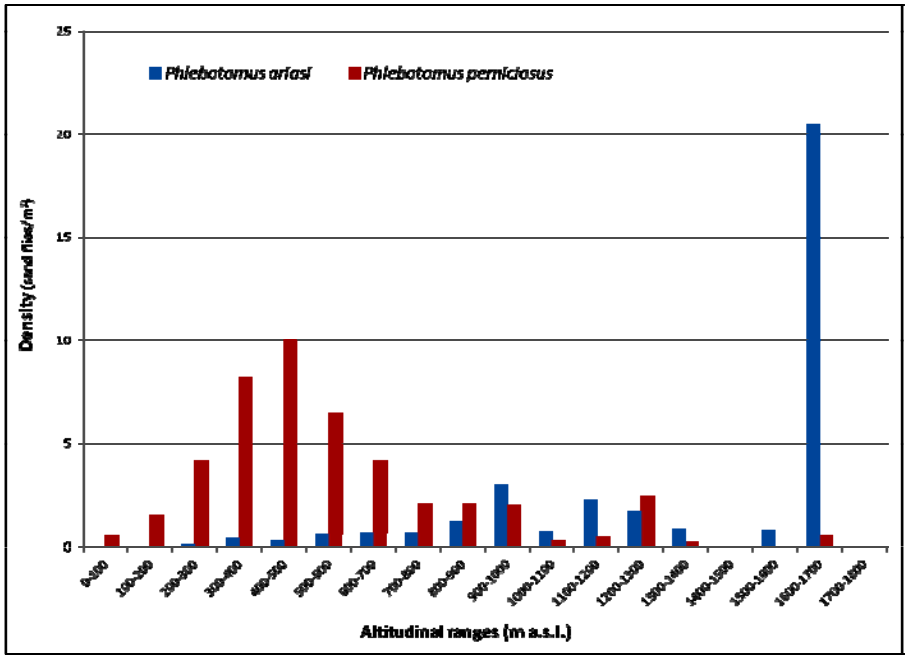

Figure 6. Density (number of sand flies per square metre of sticky trap) of *P. perniciosus* and *P. ariasi* at different altitudinal ranges in Lleida province.

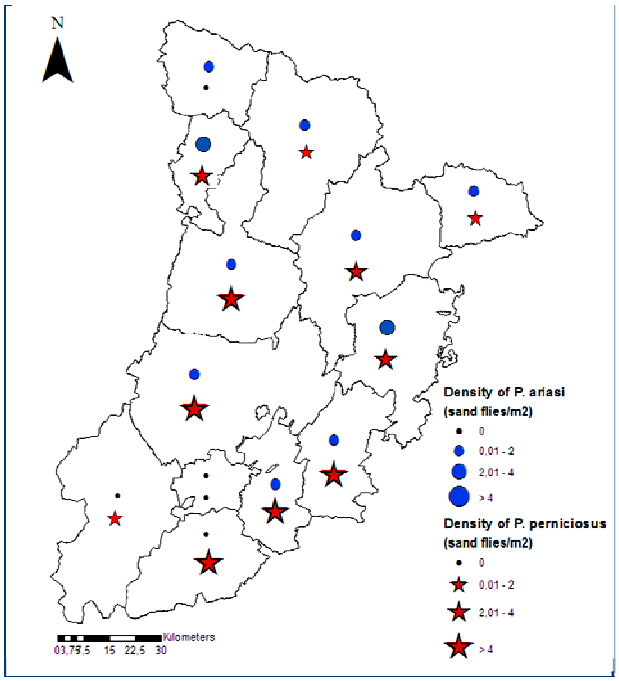

Figure 7. Global density (number of sand flies per square metre of sticky trap) of the two vector species in the different counties of Lleida province.

The density of P. perniciosus was highest at altitudes below 800m a.s.l, while the density of P. ariasi was highest at altitudes above 800m a.s.l. Both vector species have a wide distribution, but *P. perniciosus* was captured principally in southern counties and *P. ariasi* in northern ones, suggesting that each species can potentially act as the principal vector, depending on the area.

2.2. Human leishmaniosis

From 1982 to 2012, 900 HL cases were officially recorded in Catalonia [47], 39 of them in Lleida province (Fig. 8). The official data do not include the origin of the patients nor the sanitary centres where the diagnoses were made. Moreover, no data is included regarding the patients' movements within Spain and travel history to other endemic countries. The only published case of HL in the province of Lleida was diagnosed in a hospital far from the Pyrenean area and without any data of travels to other endemic regions [48]. So the presence of sporadic cases of HL is not conclusive proof of the existence of an autochthonous focus.

2.3. Canine leishmaniosis

A questionnaire on CanL, designed by the EDEN project to rapidly obtain information from veterinarians about the presence, diagnosis and

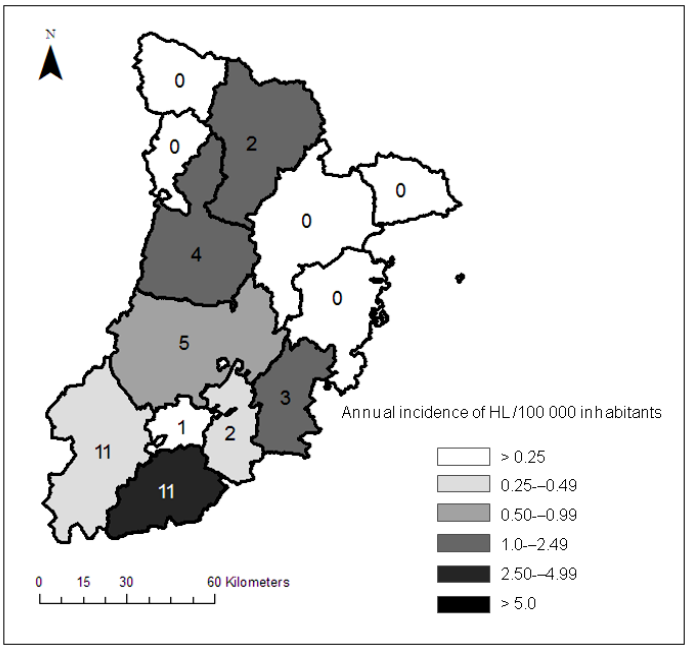

Figure 8. Distribution of human leishmaniosis in Lleida. Human data recorded from the *Butlletí Epidemiològic de Catalunya* (1982–2012) (annual incidence rate was calculated from the number of cases declared during 1982–2012 and the census of 1996). The numbers included in the different counties represent the number of human leishmaniosis cases during 1982–2012 (2012 data only for weeks 1 to 12) (updated from [49]).

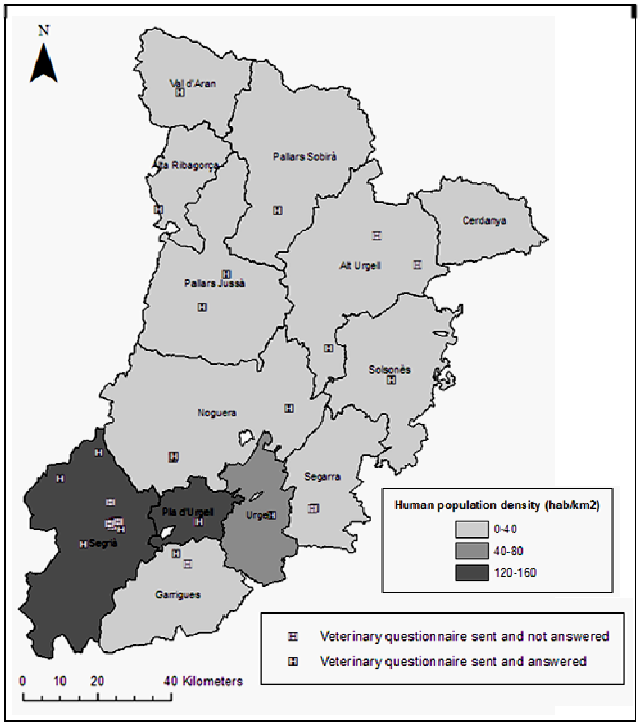

Figure 9. Study area (Lleida province and Pallars Sobirà county), surveyed points for CanL and veterinarian questionnaires [50].

perception of the disease, was applied throughout the province of Lleida (Catalonia, NE of Spain) in 2009. A total of 41 questionnaires were sent to all the veterinarians with a pet veterinary clinic (Fig. 9).

Despite the small number of questionnaires sent out, the response (78%) was considerably high, which could be related with the extent of veterinarian awareness of the disease and the widespread opinion that canine leishmaniosis has increased among patients during the last ten years (78.1%). In our study, the dogs attending the veterinarian clinics were mainly from mixed (rural and urban) areas (71.9%), reflecting that Lleida is a region composed of towns and villages with agricultural and farming activities [50]. A high percentage of the veterinarians thought CanL cases were new and acquired locally (93.8%), which plays in favour of an active focus in the region. All the practitioners (100%) recommended actively preventive measures, particularly collars and spot-on products, as in other European regions [51-53]. This could be related with the general veterinary opinion that local CanL cases had increased (78.1%) and that the infections were acquired in the area (93.8%), leading them to consider the disease as a real epidemiological problem [50].

Additionally, a cross-sectional serological study of CanL was carried out in October 2009 in Pallars Sobirà, a county of the province of Lleida located in the Spanish Pyrenees. Blood samples of 145 dogs were obtained by cephalic vein puncture (Fig. 10). Owner data, dog characteristics, geographical coordinates and altitude data were collected.

Dog samples were analyzed by four serological techniques: 1) an in-house immunofluorescent antibody test (IFAT), 2) an in-house enzyme-linked immunosorbent assay (ELISA), 3) an in-house Western Blot (WB) technique, and 4) a commercial immunochromatographic test (ICF) for the detection of circulating anti-*Leishmania* kinesin antibodies. Dogs that tested positive with at least two immunological methods were considered seropositive and probably infected [54].

Figure 10. Blood samples were obtained by cephalic vein puncture.

The long tradition of hunting among the inhabitants of Pallars Sobirà explains why a high percentage of dogs in the study were hunting dogs living in kennels in a rural environment (54%). Dogs of this kind of population have been considered as sentinel indicators of diseases of veterinary and zoonotic interest [55]. Seropositivity results are shown in Fig. 11.

The seroprevalence reported in this study (33.1%), in an area where the presence of the disease was unknown, is high considering that the highest seroprevalence value found in a known focus in Spain is 34.6% [39]. Nevertheless, this figure may be an overestimation, due to the biased nature of the sample. The analyzed dogs lived mainly outdoors in optimal conditions for disease transmission [25,26,37,56,57], due to the predominant exophilic and exophagic character of most sand fly species taking a blood meal at twilight or night [8]. The density of dogs living in kennels is another factor that facilitates parasite transmission, both directly and by vector, as previously mentioned and reviewed [36,58]. The finding of a high percentage of seropositive asymptomatic dogs (70%) prompts us to hypothesize that the emergence of CanL in Pallars Sobirà county is not very recent [50]. This idea is supported by a seroprevalence threshold usually associated with steadily established CanL foci (2.5%) [59].

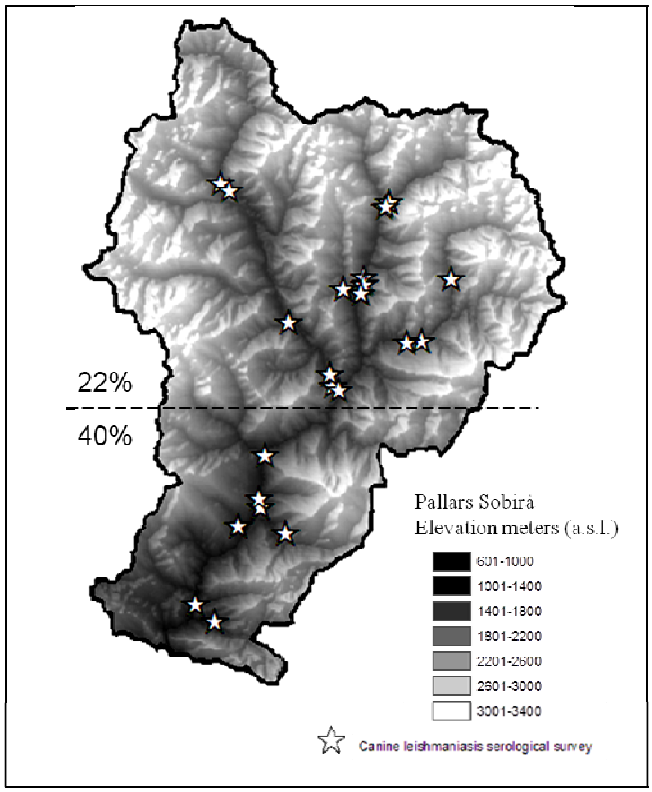

Figure 11. CanL serological survey and seroprevalence found in the north and south of Pallars Sobirà county [50].

Despite the biased results, our study of kennel dogs of local origin proved useful for detecting an autochthonous focus of leishmaniosis through the analysis of a small number of animals (145 dogs) [50].

3. Conclusion

The data on HL, obtained from the Epidemiological Bulletins, and on CanL, from the questionnaire completed by local veterinarians, suggest that both types of the disease are present in Lleida province. Nevertheless, the lack of information on the origin and travel histories of the human cases published in the Epidemiological Bulletins undermines their validity as markers of leishmaniosis distribution. The cross-sectional serological study carried out in Pallars Sobirà county allowed the detection of an autochthonous focus of CanL, which is suspected not to be of recent emergence, and confirms the presence of CanL in Lleida province. Since dogs, particularly those from rural areas, travel less than humans, CanL is a more accurate marker of the real distribution of leishmaniosis in a given territory.

The entomological surveys carried out in Andorra and Lleida confirm the presence of the two proven vectors of *L. infantum*, *P. ariasi* and *P. perniciosus*, suggesting that these territories are at risk of leishmaniosis transmission. More accurate data on the real distribution and density of the vectors are required. Studies on factors that could influence vector distribution, including climatic and environmental factors, are also necessary. The surveillance of dogs arriving from other areas where leishmaniosis is endemic is advisable to avoid or control the possible establishment of the disease in Andorra. Further serological studies on a more representative dog population of Lleida are required to know the true extent and prevalence of the infection.

Acknowledgements

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