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"Development of standardized procedures to evaluate morphological, mechanical and physical properties of nails and their alterations: pharmaceutical and cosmetics applications"

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Declaration

I, Margherita Bonetti confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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INTRODUCTION

The Nail Unit

Anatomy of the nail

The human nail is a specialized and keratinized appendix of the human body localized at the extremity of hands and feet and produced by a germinative epithelium [1]. It has a protective and defensive function against external agents, along with tactile, prehensile and aesthetic roles. In addition, based on the nail structure characteristics it is possible to interpret symptoms and signs of local and systemic diseases, as well as proceed with diagnosis and monitor of drugs therapeutic efficacy [2].

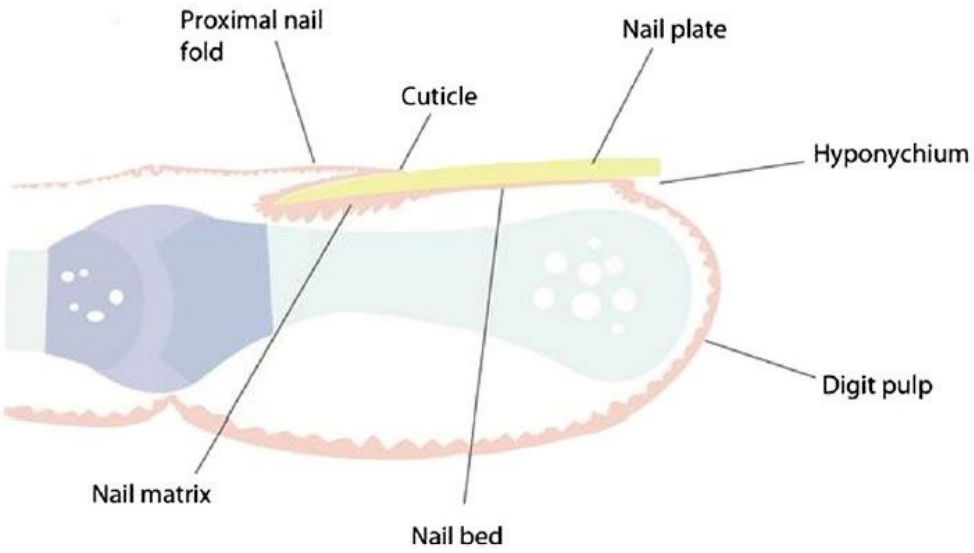

Figure 1. Sagittal view of the nail unit [1].

Anatomically the nail unit is organized as follow:

Nail plate or lamina: it is the exposed surface of the nail and is formed by keratinized dead cells and without nucleus. It fits between the nail folds and emerges from the proximal nail fold. It has a thickness of about 0,5-0,10 mm and it is composed of 80-90 % of hard keratin filaments and 10-20 % of soft keratin filaments [2]. Keratin gives rigidity to the nail structure as it contains disulfide bridges between cysteines, components of the protein itself. These disulfide bridges act as a "glue" that keeps keratin fibers together and compacts and immobilizes the structure. The level of lipid content within the plate of an healthy subject is 5% [3].

The lamina is composed of three overlapping layers:

1. **dorsal**, which is a smooth and translucent layer with the function of protecting the intermediate layer from fractures. It is characterized by indentations oriented in the direction of the growth of the nail;
2. **intermediate**, which has a fibrous composition. It is the thickest layer among the three, for this is the part most prone to fractures. Here the fibers are oriented perpendicularly to the grooves of the outer layer, therefore, in the case of fractures at this level of the nail plate, these will develop along the inner longitudinal axis of the fibers;
3. **ventral** layer, which is the innermost layer, it is flat and it is composed of cells organized more randomly.

This unique orientation and the transverse and longitudinal curvature of the plate provide structural rigidity to the nail [1].

Lateral nail folds: the lateral folds are cutaneous flaps that act as lateral edges of the nail and that allow the lamina to insert and adhere laterally to the nail unit. They have a protective action to the lamina, in fact in the event of alterations of the fold and/or in case of abnormal growth of the nail it can turn into pathologies (for example onycholysis).

Proximal nail fold: it is the periungueal cutaneous flap that covers the initial portion of the lamina. Together with the cuticle and the eponychium, it protects the melanocytes of the matrix from irritants, solvents, microorganisms, and

ultraviolet radiations, thus avoiding permanent damages that would affect the growth of the nail or causing systemic disease.

Eponychium: it is part of the proximal nail fold and is composed of live tissue, thus it is perfused with blood vessels. It acts as a sealant and a barrier against the entry of infectious organisms into the germinative matrix. The destruction of the eponychium can lead to the onset of inflammation and lead to the paronychia, an infection of the periungual tissue.

Hyponichium: is the distal edge of the nail and lies below the free edge of the lamina, which is separated from it, and so it appears colorless (white).

Peronychium: is the tissue that encloses the nail plate.

Nail matrix and lunula: the matrix represents 15%-25% of the internal tissue of the nail unit and it is the main responsible for the formation of the nail plate, as it produces keratin and new cells. Clinically, the element of the distal matrix, the lunula, is visible in some people, while in others it can be hidden by the proximal fold. The lunula is a whitish half-moon positioned in the proximal zone of the lamina. The matrix is found in the nail groove and, in addition to the blood vessels, contains lymphatic vessels and nerves. It produces keratin thus allowing the growth of the nail plate of which determines thickness and size. As the keratinocytes located in the matrix produce new cells, they move to the distal zone of the lamina increasing nail length. Growth, with a rate from 0.5 to 1.2 mm per week, is influenced by several parameters such as fever, drugs, age, disease, temperature and physical stimuli. To clarify the nature of the nail differentiation pathway, Takashi Kitahara and Hideoki Ogawa [4] examined the expression of keratinized cells in human nail in comparison with cultured cells obtained from the bovine hoof matrix. In this study, they were able to demonstrate that the human nail matrix is characterized by the different and separate localization of cutaneous-like and hair-like keratins, except for the apical matrix in which both types of keratins are co-expressed simultaneously. Using indirect immunofluorescence techniques it has been discovered that the matrix is composed of skin-type cells as well as hair-type cells and another cell

type that expresses both keratins specific for differentiation, thus suggesting that the matrix of the adult nail develops following multiple pathways of differentiation, with some cells in an intermediate stage between skin and hair differentiation. Moreover, the fact that cultured cells from the ventral zone of the matrix express cutaneous-like keratins (Kl/K10) and hair-like keratins indicates that these cells develop through in vitro differentiation of both skin and hair type. The multiple differentiation patterns that develop in the matrix of the nail are attributable to the possible cell diversification observed in cells grown in vitro.

The lower layers of the matrix also contains melanocytes, and this can lead to observe different degrees of pigmentation of the nail itself. The matrix can be divided into two main areas: the proximal matrix, which produces 50% of the superficial cells of the lamina, and the distal zone, which produces cells of deeper and less resistant layers. In the case in which the matrix suffers lesions it is possible to observe anomalies in the growth of the nail.

Cuticle: it is a layer of epidermis that extends from the proximal nail fold to form the protective layer of the nail matrix. Often during manicure this layer is eliminated, causing inflammation and decreasing protection against pathogens.

Nail bed: it represents about 75%-85% of the inner tissue of the nail unit [5]. It extends from the distal unit of the lunula to the hyponychium. The avulsion of the nail reveals a pattern of longitudinal epidermal crests extending up to the lunula, finding complementary grooves in the upper part of the lamina resting on them. The classic pink coloration of the nail bed is provided by vessels and capillaries that flow in the same direction as the crests.

The nail bed has a low proliferation rate and does not produce terminal differentiation keratins present instead in the skin. This is reversible: when onycholysis occurs (which is the detachment between the nail bed and the lamina), the production of these terminal keratins would start, going to form a granular layer and causing the loss of the crests on the lower part of the lamina. These three elements, the lack of terminal keratins, the lack of a granular layer and the wavy surface of the nail plate, seem to play a fundamental role in the

adhesion between the nail plate and the nail bed. As part of the hands, the vascular and nervous networks are fine and complex [1]. The radial and ulnar arteries provide nourishment to the palmar arches, the superficial arch and the profound arch, acting as an anastomosis between the two vessels. From these two arches branch out the blood vessels that align at the level of the phalanges. Four arteries are observed per finger, two on each side. Palmar digital arteries provide the highest blood content to the fingers and receive the blood itself from the palmar superficial arches. Although coupled, generally one of the two arteries is dominant. Dorsal digital arteries, on the other hand, are small and arise as branches from the radial artery. The venous drainage of the fingers is formed by a deep system that corresponds to the arterial network and a superficial system that is composed by the dorsal and palmar digital veins. These generate a network of blood vessels often in evidence on the back of the hand. The soft periungual tissues are innervated by the dorsal branches of the coupled digital nerves. Such innervation is fundamental for the tactile function of the hand and it is present mainly at the level of the dorsal part of the finger, especially in the nail folds. For this reason, loss of sensory sensation can be observed in patients with surgically operated nail.

Biochemical composition

The main chemical compound constituting the human nail is keratin, a scleroprotein containing a large amount of sulfur [6]. Studies using indirect immunofluorescence as the main technique of investigation have shown that the nail unit composition includes three cell populations: cells that express skin keratin or hair keratin and cells that express both types of keratin. The filaments of alpha keratin (helical structure) interact with the cysteines of the non-helical keratin dispersed in the matrix, directing perpendicularly to the direction of growth of the nail and parallel to the outer side of the nail. This alignment of the keratin filaments and the strong adhesion between the cells contribute to the hardness of the nail itself [7] [6].

The nail contains different amounts of amino acids that form the polypeptide chains present in keratin. In general, the lamina is mainly composed by an abundant percentage of glutamic acid, alpha cysteine, arginine, aspartic acid, serine and leucine. The lipid content of the nails is usually below 5 %,

motivating the resistance to water of the nail plate. Glycolic and stearic acids are the main lipid types found in the lamina [5] [2].

The standard water content of the nails is around 18%. However, it may vary depending on the weather conditions: the increase in temperature increases water diffusion ability, while changes in the humidity of the air could decrease it. Some studies have shown that nails are similar to hair concerning the water content behavior as they are much more water permeable than the stratum corneum of the skin [5].

Many trace elements are present, the most abundant are zinc and iron; a deficiency of the latter has shown to be correlated with thinning and brittleness of the lamina [8].

In addition to iron and zinc, other mineral elements such as calcium, silicon, selenium and copper could be found. Contrary to popular belief, calcium content does not correlate with the hardness of the nail plate and it occupies only 0.2% of the weight of the nail [2]. It could be found in larger amounts in male subjects and in general in older individuals. Nevertheless, a calcium deficiency may lead to leukonichya, which is a condition characterized by an excessive keratinization of the nail matrix [5].

Selenium is a nonmetallic element found in traces in our bodies. It acts as an important coenzyme as it intervenes in antioxidant enzyme systems such as glutathione peroxidase.

Silicon has been shown to improve the conditions of nail fragility and psoriatic nails.

Several studies have been performed to evaluate the role of Vitamins in nail properties and pathologies [5] [9] [10] [11].

Vitamins like Biotin (or Vitamin H), Vitamin E, Vitamin A, Vitamin D and Vitamin B12 play an important role in the nail. Biotin is mainly used in the treatment of the Boucher-Neuhäuser syndrome as it improves the hardness and thickness of the nails. Vitamin E is used in the topical and oral treatment of the Yellow Nails Syndrome. Vitamin A plays a double role in the health of the nail because on the one hand it is able to improve the condition of psoriatic nails and nails affected by congenital paronychia, on the other an excess of Vitamin A can lead to fragility, onychoschizia, onychomadesis and onychoressis. Vitamin D

is indicated in the treatments of psoriatic patients. Finally, a deficiency of Vitamin B12 can lead to hyperpigmentation of the nail plate.

Physical Properties of Nails

The strength and the physical characteristics of the nail plate are to be attributed both to the composition and to the shape of the nail plate itself. The most influential features are the double curvature along the longitudinal and transverse axes, which allows to support the rigidity of the lamina, and the flexibility of the ventral portion of the lamina [8].

The nail plate is in fact hard, but also elastic, flexible and resistant. These properties derive from the anatomical characteristics of the different layers of keratinocytes that compose it.

Some studies show how two portions of lamina can be distinguished: a superficial portion, which originates from the proximal matrix, formed by flattened cells, very adhered to each other, and a profound portion, which originates from the distal matrix, formed by less adhesive cells and having jagged outlines. The first layer of the lamina is hard and sharp, but at the same time inelastic and fragile, while the deepest part is flexible and elastic [12]. Electronic scanning microscopy studies however, clearly shows a three layered composition. The dorsal layer appears as constituted by flat sheets superimposed on each other, oriented in the plane of the nail. The intermediate layer is instead more fibrous, with the fibers oriented transversely, in parallel with the outer edge of the nail. Finally, the thin ventral layer is similar to the dorsal layer.

Hydration

The hydration of the nail is considered the fundamental and most influential factor to characterize all its physical properties. It shows a strong dependence on temperature and relative humidity of the air. The diffusion of water through the foil is also influenced by the air currents of the surrounding environment [13]. The only investigation technique used in vivo on nails is NIR spectroscopy; instead the classical techniques applied to the stratum corneum based on measurement of quantities such as conductance or capacitance are not used, because they are strongly influenced by the contact area between

the probe and the nail plate. From the data obtained with NIR spectroscopy, it is inferred that the average water content of the nail plate is around 7-25%. In fact there are many elements that influence this content, increasing or decreasing it. As can be seen from studies conducted with Raman spectroscopy, water is mainly present in a bound form, ie in heterogeneous structures formed with other molecules through hydrogen bonds [14]. In general it can be said that the water content of the nail plate decreases during the summer season ($P < 0.05$), when it is in the presence of cracks, the decrease in environmental humidity and with increasing age [15]. Regarding the relationship between the water content and the mechanical properties of the nail plate, the results of different bending tests show that a water content of more than 20% weakens the nail, making it extremely soft, while a content of less than 10% increases the probability of fracture [15]. The change observed in the physical properties of the nail, in relation to its level of hydration, reflects the alterations occurring in the molecular structure of proteins. We know, in fact, that the keratin of the nails belong to a group of hard keratins that also include keratin of the hair and the hoof. These consist of two phases: a fibrous phase and a matrix, protein and amorphous phase, linked to each other. The fibrous phase consists of α-helix proteins with a high molecular weight. These are wrapped together in stable microfibrils and are found in aggregates. The sulfur content of microfibrils is lower than that of the matrix proteins (5.5% compared to 20.3%). The matrix phase is instead stabilized by cysteine-cysteine disulfuric cross-links and by secondary interactions, including Van der Waals forces, hydrogen bonds and ionic interactions. Because of the large presence of hydrogen bonds, the matrix phase is, in conclusion, more sensitive to the effects of hydration than the filaments [16]. Compared to the skin, the water content of the nail is lower; this because the proteins in the nails and in the hair are more folded, while in the skin the looser structure allows a greater content of free water. This contrasting level of hydration allows metabolic processes in the skin that are impossible in the nails [14]. The most widespread and widely accepted method to estimate the barrier function of the integumentary apparatus is the determination of trans epidermal water loss (TEWL, TransEpidermal Water Loss) with an evaporimeter. As for the epidermis, also for the nails it is possible to measure

the transungual water loss, called TOWL (TransOnychial Water Loss). The mean TOWL value found in healthy adult subjects settles at around 13 g / cm2 / h. This rate of water loss is significantly different from the TEWL typical of the back of the hand, with an average value of 9.1 g / cm2 / h. TOWL decreases with age and does not appear to be influenced by the thickness of the nail plate [17]. In the case of diseased nails, there is an opposite behaviour with respect to TEWL, as in pathological patients a lower TOWL is always observed [18]. Changes involving the fingernails associated with aging are very common in both the sexes. They typically cause discolouration associated with a change in colour from pinkish-white to grayish-yellowish. Perturbations also occur in the edges of the lamina, in the groove of the longitudinal furrows, in the form of the curvature (less concave), in the thickness and roughness of the surface of the nail plate. Structural changes can result in brittle nails and in various pathological conditions. The most common disorders of aged nails are simple fungal infections. Although these do not constitute a serious medical concern, they represent a cosmetic problem that can be extremely influential especially in female patients. Women typically show a greater concern regarding the cosmetic appearance of the nail compared to men of the same age group.

Hydration of the nail plate plays a critical role in its physical behavior. Nails show high porosity and the water content of the nail plate varies easily. In fact, under normal conditions the water content reaches 18%. A content higher than 25% makes the lamina too tender and flexible, while a water content below 16% makes it brittle. Moreover, when the percentage of lipids decreases, the hydration of the nail is more difficult and there is a tendency to fragility [8] [19]. In a study conducted by Laura Farran et al. [20] it has been shown that increasing the immersion time in water also increases the number of flexions needed to break the nail plate. This is due to changes in the keratin matrix. Torsion experiments applied on human nail portions showed how the physical characteristics of the nails vary under conditions of high hydration and how, once returned to normal conditions of humidity, the lamina regains strength as keratin filaments rebuild very strong bonds between them.

In this study, three controlled relative humidity (RH) levels have been tested: RH=0%, RH=55%, RH=100%. Nail plate deformation (stress), as a consequence of an applied force (strain) varies in relation with RH levels [20].

Hence, after the application of an external force, initially an elastic deformation could be observed, reversible until the yielding point, after this the deformation become plastic and thus irreversible. The RH level strongly influenced internal bonds within the lamina, with a consequent variation of the yield point as the plate is more flexible. With a RH below 55% the nail plate is more fragile. Above 55% of RH the plate is more easily damageable if put under torsion.

Consequently, it is possible to say that hydration is a critical parameter affecting not only physical properties of nails, but also permeability properties and hence active substances permeation through the nail barrier.

In fact, a recent work by Elena Cutrin Gomez et al. [21] shows how low is the porosity of a dried nail plate, critical condition for defence from xenobiotics. Hydrated nails show a porosity increase from 6,97% to 15,24% and an increase in permeability (fluid permeation).

Healthy nails show a reduction in porosity from extremities to the lamina centre, preventing the absorption of any elements. With an increase of the hydration level, hydrophilic proteins expand their structure, with a reduction in disulphide bonds between cysteine inducing a reduction of nail plate firmness. In this way, the lamina act as an hydrogel where pores and water create a network which allows molecules movements through the plate.

Mechanical Properties of Nails

Despite the in-depth knowledge available in literature regarding the anatomical structure and the development of the nail unit, relatively few studies have been performed to correlate the structure with its mechanical behaviour. Considering the tasks for which the nail unit has evolved, it must therefore resist forces that push it and bend it upwards. The strength of the nail depends on its constituents, on the shape of the cells that compose it and their specific disposition. One of most significant factor is the double curvature along the longitudinal and transverse axes which, as already described above, allows to support the firmness of the lamina, and the flexibility of its ventral portion, which increases its deformability potential [22]. The α-helix keratin strands interact with the non-helical keratins of the matrix rich in cystine, orienting themselves perpendicular to the direction of nail growth and parallel to the free edge. This alignment of the keratin filaments and the strong adhesion of the cells of the

nail plate to one another strengthens the nail, especially in relation to the forces applied along the longitudinal axis [23]. To evaluate all these properties, various mechanical tests have been performed including tensile tests, cutting tests on the entire nail and on the individual layers that compose it and deflection test. The results of the tensile test show significant differences in the ease with which a break can propagate in different directions. When trying to tear the nail with a longitudinal orientation along the nail bed, the required force appears to be high and the break never propagates longitudinally. On the contrary, a break propagates easily with transversal orientation through the nail, following the curvature of the lunula. The results of the cutting tests allow to understand why there are such differences in the fracture properties in the two directions. In fact, the central section of the nail is about twice as strong as the longitudinal plane compared to the transverse plane. The results of the tests on the individual layers allow to locate the anisotropy of the nail in the intermediate layer. This is about 4 times more resistant in the longitudinal plane rather than along the transverse plane. In contrast, both the dorsal and ventral layers showed an essentially isotropic behaviour, with less resistance along the longitudinal and transverse axes. The results of the tensile tests show that the nails possess a remarkable ability to limit the propagation of the breaks longitudinally, along the nail bed. First of all, this is because human nails are made of an overall resistant material: the resistance is about 3 and 6 kJ m-2 respectively along the transverse and longitudinal direction. The ease of spreading a break in the transverse direction seems to be correlated with the anisotropy of the nail plate. A break, in fact, to spread transversely requires half the energy necessary for longitudinal propagation. This anisotropy occurs because the composition of the nail is dominated by the thick intermediate layer, consisting of long and narrow cells, as mentioned previously, oriented parallel to the lunula and side by side to each other. To cut such a layer transversely, a quarter of the energy required to cut it longitudinally is required. This happens because a transverse cut must only separate the cells from each other, while a longitudinal cut should break the cells. The anisotropy of the intermediate layer is, to some extent, opposed by the two thin dorsal and ventral layers, isotropic and made up of tile-like cells, with randomly oriented keratin fibers. The phenomenon is more accentuated towards the tip of the

nail, where the outer layers become relatively thick. A coarse function covered by these layers is to increase the overall strength of the nail, placing one's own cells next to those of the intermediate layer. However, the resistance of the nail unit as a whole is increased due to a volume effect: unlike hair cells, in fact, the nail cells have particularly thick cell walls [24].

Numerous factors have been indicated as responsible for the firmness of the nail plate due to their presence or absence. The proteins are arranged according to a highly ordered pattern, which certainly helps to increase the resistance and firmness of the nail plate especially compared to what is observed with the skin [22]. Unlike bones, the firmness of the nail plate does not depend on calcium levels. The disulfide bridges of the cysteine, which constitutes the hard keratins of the nail, and all the folding of the secondary structure of the proteins allow a stabilization of the structure that, to a lesser extent, can also be found in the stratum corneum. What changes, however, is the geometry of the fold that is gauche-gauche-gauche in the nails (and in the hair) and gauche-gauche-trans in the stratum corneum. The folding gauche-gauche-gauche is more stable. Desmosomes could also play a role in the cohesion of the nail plate and, consequently, in its rigidity. The physical properties of the nail plate, such as its curvature and the orientation of its fibers, perpendicular to the direction of the growth of the lamina, are factors that can decrease the fragility of the nails. Finally, the water content has also proved essential to increase the flexibility of the nail plate and the elasticity, thus helping to reduce its fragility [25]. In fact, it is shown that the effective elastic modulus (independent of the size of the nail plate of a particular species) depends on the level of hydration of the nail and not on its natural curvature. Another factor that can influence the effective elastic modulus is contact with detergents, organic solvents or oils [13].

As stated before, hydration has a huge influence on nail physical properties.

In the study carried out by L. Farran et al [20], there have been used 60 nail clipping samples (at leat 3mm x 9mm) from 50 young healthy volunteers. As already reported above, L. Farran at al. demonstrated how tensile properties of nails changes due to relative humidity condition variation (RH0%, RH55%, RH100%). To control and to assess RH environment around the samples and then samples humidity content, nail clippings were stored in boxes with

saturated saline solutions. After storing the samples in these boxes for 48hours in order to condition them to the environment, they have been inserted in a custom made instrument consisting in clamps made up with metallic fibres and paper in order to prevent any fall out. The assay has been performed keeping the keratin fibres of the samples parallel to the traction axis.

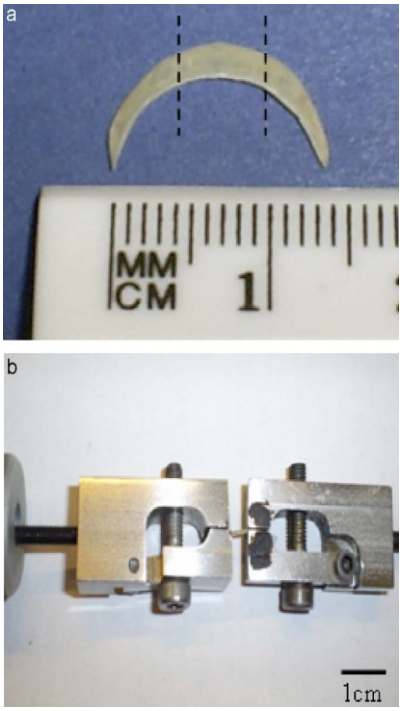

Figure 2. Nail samples used for tensile testing: (a) a typical nail sample and (b) a nail sample in the metal fibre grips [20].

Torsion experiments performed on nails have demonstrated that the shear module of the lamina change drastically due to RH conditions, suggesting the critical role of humidity in alteration of the fracture properties of the keratin network. The deformation has shown to be reversible, suggesting an autonomous reforming of the bonds involved in the keratin network of the lamina.

Nail permeability

The permeability of the nail plate is a fundamental element for the penetration of drugs for topical use, which should migrate from the dorsal to the ventral portion. Due to its biochemical composition and structure, the nail plate is an amazing protective barrier, thus challenging for drug delivery. Active

ingredients diffusion is strongly limited in comparison to other tissues such as skin [7].

However, topical therapies are a great treatment opportunity when dealing with nail diseases: they are not invasive, drugs are administered directly on the site of action, they hardly show systemic adverse effects, decreasing possible complications for patients under multiple treatments, and the cost are usually lower in comparison to systemic therapies. Since these treatments are designed to be used on the nail plate, their efficacy need to be enhanced, ussually applying the following criteria [21]:

1. using potent active molecules, where a low dose is necessary to be effective;
2. active ingredients with physico-chemical properties compatible with ungueal permeation;
3. using carriers able to facilitate active ingredient absorbance;
4. appropriate formulation, easy to be used, allowing drug permeation and a long contact-time with the nail plate.

In general, topical absorption of drugs through skin/nail barrier is regulated by the Fick law of diffusion.

The factor which affect a molecule permeation through a barrier are:

- Molecular weight of the compound, which is inversely proportional to the diffusion rate: the more the molecule is big the harder will be the permeation through the nail keratin network.
- Repartition coefficient: several studies concluded that the nail plate behaves as a concentrated hydrogel. Moreover, it has been shown that hydration of the lamina facilitates molecules diffusion through it.
- Medium or vehicle, which in this case could be associated to the formulation as a whole: water-based vehicles are less suitable for topical application, since they are easily removable and dryable. Moreover, they usually will not adhere to the lamina at the same extent of an organic solvent-based formulation. However, as already stated hydration strongly contribute to molecules permeation through the lamina, so the balance among this factor is still unclear.

- Degree of ionization of the molecules and pH of the medium: formulation pH affects ionization degree of acidic and basic compounds, having an impact on lipophilic/hydrophilic properties and on the solubility degree of the molecule and thus affecting drug permeation and interaction with the lamina [26].

Since nail plates are only available to a certain extent for the preclinical development of topical drugs, it is of interest to calculate the expected permeability coefficient of the nail using an in vitro model.

In vitro model

In the last 20 years nail in vitro models have been meticulously study and characterize in particular in relation to permeability assays.

Permeability studies have been performed using modified Franz cells apparatus, but also analysing nails swelling and drugs absorption within them. The in vitro models more frequently used are nails from cadavers, nail clippings from human volunteers and bovine hoof membranes [27].

Hoof membranes are generally obtained from fresh slaughtered cows: after removal of adjacent tissues the hoof is usually soaked in water and then membranes are dissected using a microtome to obtain the desirable thickness. However, it often unclear which exact procedure has been used for the production.

Mertin and Lippold performed several studies for the evaluation of the permeation properties of hoof membranes in comparison of those of human nails. In the first study, they evaluated homologous nicotinic esters permeability [28]. The partition coefficient of the esters ranged from 7 to > 51,000. However, despite the wide range tested, the permeability of nail and hoof to these compounds did not increase with increasing lipophilicity of the molecule. In another study, the same authors compared the permeation of chloramphenicol through human nail and bovine hoof membrane from three vehicles saturated with the drug: phosphate buffer, n-octanol and medium-chain triglyceride [29]. There was a 10-fold difference in the chloramphenicol solubility in the vehicles. However, the maximum fluxes from these vehicles through each membrane were nearly equivalent after the data were

normalized to the thickness of the membrane. The investigators concluded that when swelling of the nail plate or hooves is not altered by the vehicles, the maximum flux of a drug is independent from its solubility properties in the vehicle and is equivalent as that of the saturated aqueous solution. In the same study, various nail lacquer concentrations (0.5%-20%) of chloramphenicol were tested for both human nail and hoof membrane permeation. Bovine hoof permeability was found to be 54-fold higher (nail lacquer) and 65-fold higher (saturated aqueous solution) than that of human nail, after adjusting membrane thickness. This difference was attributed to the different chemical composition of the two membranes, (i.e., the hoof has only one-half of the half cystine content as human nail). Therefore, membrane with higher sulfur content was both tougher and a stronger barrier.

In another study, Mertin and Lippold investigated the effect of the molecular weight on permeation through human nails and in vitro model [30]. They showed that the difference in permeability between the nail and hoof membrane increased with increasing permeant molecular weight. Hence, there is a chance of overestimating drug penetration into nail using the hoof membrane as a model.

To summarize, with these three different works the authors showed that hoof membranes are a suitable in-vitro model regarding the prediction of the permeability of the nail plate. Although the permeability coefficients of the nail plate and the hoof membrane differ from each other, they proved that the bovine hoof membrane may serve as an appropriate model for the nail, since they both behave as hydrophilic gel membranes. Moreover, the logarithms of their permeability coefficients are related with a linear correlation, therefore it is possible to directly derive healthy nail permeability coefficient after experimental determination of the hoof membranes one.

Monti et al. in 2011 [31] not only confirmed the previous findings but they also shown an equivalence between bovine hoof membranes and infected toenails in the assessment of ciclopirox permeation/retention.

However, it is critical to pay attention when using hoof membranes as a model for the human nail in permeability assays. Hoof has the tendency to be more permeable in comparison to human nail: it is considered that its keratin net is

less dense and, if incubated in water, hoof membranes swall up more (36% in comparison to 27% of human nails clippings) [27].

As stated above, hoof amino acidic composition is different, with a significant lower content of alfa-cysteine and disulfide bonds than human nail. As a consequence, hoof could be more sensitive to those compounds which breaks disulfide bridges and are studied as potential permeability enhancers. In these cases, the improvements obtained with hoof membranes could be less of the improvements obtained for human nails [26].

Human nails and animal hooves are made by the same keratin type (alfa-keratin). The main differences include the relation ships among amino acidic groups and their internal structures. Nevertheless, both human nail and animal hooves has a hair-like keratin, well known for the high sulfur content [32].

In addition to hoof membranes, other animal derived in vitro model have been used. For examples, Myoung et al. have used pig's clogs to evaluate permeation properties of the antifungal drug Ciclopirox.

In 1997 Mertin and Lippold have examined in vitro permeability of several antimycotic agents using membranes derived from porcine hooves, employing diffusion cells for the assay. Selected drugs were suspended in ethanol 42% (v/v) which has been used as vehicle in the donor compartment too. The maximum flux obtained through the membrane for the 10 antimycotic analysed has been calculated based on their permeation rate through the barrier and on their water solubility using the equation described above for bovine hoof membranes (logPN=3.723+1.751 logPH).

Then, a coefficient of efficacy against onychomycosis has been calculated taking into account the highest flux value and the MIC value of each drug (minimum inhibitory concentration). Using experimental data from Mertin and Lippold study, several mathematical models have been developed to predict human nail permeability for a specific compound.

In summary, several and accurate validation studies, which considered molecules with different molecular weight and solubility characteristics, have helped to highlight the functional similarities and differences between human nail and the in vitro models based on animal hooves. However, available data are still not sufficient to have a general consensus and to determine an exact correlation between them [33].

An alternative to animal models are human nail clippings from healthy volunteers, whic have been used in the past as a model for human nail in permeability studies. They are easy to obtain, but the surface available is really limited, making it not the best model for nail products assay. Moreover, this model still needs a validation study to compare it to the cadaver nails model [33].

Nails extracted from cadavers have also been used to perform ex vivo assays to assess permeability of molecules and products through the nail barrier.

In order to develop a novel ex vivo model, to mimic physiological conditions as close as possible also intact human fingers from cadavers have been employed, which could replicate in vivo conditions making possible preclinical evaluation of permeation of a novel product through the nail. This model has been used to assess iontophoretic release of the antimycotic drug terbinafine [34].

Keratin films could another option for reproduction of the nail barrieir in vitro. Lusiana et al have developed a model to simulate the nail plate which is composed by keratin fibres extracted from human hair [32].

In fact, keratin is not a single molecule: as stated before, it is composed by a complex mixture of proteins. It is insoluble in ordinary solvents such as diluted acids, alkaline vehicles, water and organic solvents.

To extract keratin, the use of reducing agent is necessary because the original structure is hard to obtain due to the strong presence of disulfide bonds.

The Shindai method, which does not employ detergents, provides a large enough amount of extracted proteins for the analysis and it allows not to rely on hydrolysis. The alfa-keratin which composes human hair can be found also in the nail plate and several studies have shown similarities between hair and nails physico-chemical properties.

One of the main aim of Lusiana et al. work was to extract keratin from human hair by the Shindai method and re-assemble it in a film capable of mimic human nail plate. The properties of the model have been examined studying permeation of several markers and assessing nail permeation enhancers. For comparison, bovine hoof membranes have been used (ndr. Bovine hooves could easily found and are usually cheap or free of charge since they are slaughterhouse waste products, while human nail availability is really limited

and expansive). The three markers selected for the assay were: sodium fluorescin, rodhamin B and fluorescine isothiocianate dextrane. The first molecule represent hydrophilic molecules, the second the lipophilic ones, while the third has been used as a model of molecules with high molecular weight. Three permeability enhancers have also been selected: urea (acting through an augmented hydration of the nail plate), tioglycolic acid (acting through the breakage of the disulfide bonds of the keratin fibres of the lamina) and papaine (endopeptidase which hydirolize disulfide bonds).

For the permeability assay modified Franz cells apparatus has been used. Both donor and acceptor compartment have PBS as the main vehicle. Moreover, in the donor compartment markers were dissolved in the PBS.

The results obtained, Lusiana et al. concluded that keratin film could represent an appropriate in vitro model for permability assays due to its mechanical stability and water resistance properties, similar to the human nail, in particular for hydrophilic substances. However, the interpretation of experiments conducted on lipophilic substances and permeability enhancers is critical, because keratin films could overestimate the permeation rate.

Cellular culture in vitro is a well known and well accepted method to assess preliminary investigation on cell behaviour. It is adequate for systematic and quantitative studies and unlike in vivo studies, tissue models in vitro could be strictly controlled, with a high reduction in time and cost of the research as well as a reduction in animals used.

Ideally, an in vitro model should be resemble the real-life condition as much as possible, emulating the specific dynamic micro environment of a real tissue.

In vitro test, physiological barriers represent a key point of access for transport and absorption of endogenous and exogenous substances. In fact, chemicals introduced in the systemic circulation are carried through the biological barriers such as cutaneous epithelia, gastro enteric or bronchial tracts, which could modify their kinetic and metabolic profiles. Moreover, these compounds could interact with cells and tissue and modify cellular function, for example inducing a permeability increase of the barrier. In general, for all these models the cells culture method is always the same. Basically the cells which composed the epithelium are seeded on a membrane, which is then position between two different compartment of growing medium, as if it was a modified Franz cell

apparatus for cellular culture [35]. It is possible to use this technique to simulate the nail plate starting from epithelial cell culture.

Nails overhydration is one of the most used in vitro methods to assess drugs permeation through the nail plate using modify diffusion cells. It is based on the same concepts as skin permeation assays, where permeation rate is measured distributing the drugs in several points on the ventral portion of the lamina and calculate the rate of permeation of the drug through the plate. Hui et al. Have slightly modify this approach using a cotton pad soaked in saline solution to ensure humidity (but not to a saturation point) and hydrating the nail during all the experiment. The support was designed as follow: a diffusion cell and a Teflon chamber were used to block every nail mimic the physiological conditions with a cotton pad positioned on the chamber to provide humidity to the nail plate. As already mentioned, hydration could increase pores dimension of the lamina, promoting transungual permeation of actives. Moreover, it is possible to observe in our everyday life daily how easily nails could become softer, more flexible and elastic when soaked in water. Thus, all the studies results which do not take into consideration this factor should be evaluated again [33].

In vitro data obtained by the use of modified diffusion cells, nail clippings from volunteers, human nails from cadavers and bovine hoof membranes as a model for the nail plate required a comparison with in vivo studies derived from works with radioisotope and atomic mass spectroscopy [33].

One of the main limits of the in vitro model described is that has been assumed permeation as a passive process, not considering vital components to influence permeation through the nail barrier (e.g. blood flow) [33].

Nail Diseases

Nail unit anomalies include a wide range of conditions from easy ones, such us abnormal pigmentation of the plate in heavy smokers, to painful and debilitating conditions where the nail could be dystrophic, ipertrophic, inflamed or infected [36] [37] [38]. These conditions will affect patients not only physically but also psychologically, since they have been shown to have a strong impact on patients quality of life and every-day life activities. Moreover, nail diseases are well-known to be hard to cure, requiring long term treatments

and showing high frequency of relapse episodes. Oral therapy could cause systemic side effects and drugs interaction problems, while topical treatments are limited by nail plate permeability barrier.

Brittle Nail Syndrome

Brittle nail syndrome (BNS) is a common nail disease observed in up to 20% of the population with women affected twice as frequently as men [39]. It is characterized by the impairment of intercellular adhesive factors with the lamellar splitting of the distal portion of the nail plate, caused by internal and/or external factors [36]. Along with its manifestation as a primary disease, idiopathic in the majority of cases, BNS is also a common secondary manifestation of several skin diseases like psoriasis, lichen planus, and alopecia areata [40]. As stated in a recent review published by M. Chessa et al. [3] Optimal management requires the treatment of the primary cause of BN when present, protective measures and bio-mineral supplementation. In addition, there are several products which could be considered to reduce the impact of the disease. On the market, there are several nail lacquers available, aiming to restructure nails affected by fragility. These products are commonly known as nail hardeners or nail strengtheners. However, there is still a lack of clinical studies to support products efficacy. The situation is even more critical when looking for in vitro studies to support the efficacy of this products.

Onychomycosis

It is estimated that nearly a billion people have a skin, nail or hair fungal infection, many tens of millions are affected by mucosal candidiasis and more than 150 million people have a serious fungal disease worldwide [41]. Some of these fungal infections can have a major impact on people’s lives or can be fatal, if the infection is not treated effectively.

Onychomycosis, fungal infection of the nail, is the most common nail disorder affecting the nail unit. In children the prevalence (0.44 – 2.6%) is lower in comparison to adults and older people (20.7% in adults aged over 60 years old) [42]. Risk factors of onychomycosis include: aging, trauma, diabetes, immunosuppression, psoriasis and family history of onychomycosis [43]. Symptoms of onychomycosis includes discoloration of the nail from white to yellow/green, nails become brittle and are more prone to break, unpleasant

smell, thickening of the nail plate and dry/scaly skin around the infected nail. Pain is less common, however it may vary depending on the severity of the infection as well as the time the infection has been left untreated. The longer the duration of the nail being untreated, the more likely the infection will worsen and may get inflamed.

Fungal infections can be associated with three types of microorganisms: molds, yeasts and dermatophytes. The latter are the most commonly encountered in temperate zones and in about 80% of cases the fungus involved is *Trichophyton rubrum* [44]. However, fungal infections actually depend on the geographical area [45]. In fact, in temperate countries *Candida albicans* infections have been shown to be recurrent, since it can be isolated as frequently as dermatophytes [46] .

In general, the main pathogenic fungi are divided into dermatophytes and non-dermatophytes. "Tinea unguium" indicates precisely the dermatophyte infection of the nail plate, from the Latin name of the part of the body involved, while the term “dermatophytosis” is used to describe infection by members of the genera *Microsporum*, *Trichophyton*, and *Epidermophyton.* The species that most often cause onychomycosis in North America and in most part of Europe are *T. rubrum*, *T. mentagrophytes*, and *Epidermophyton floccosum*. Dermatophytes are hyaline-setted molds: the hyphae of these mycelial organisms penetrate the stratum corneum of skin and nails. Fungal cells produce keratinolytic proteases that provide them with an entry way into the host [47]. Some dermatophyte species, which are basically soil saprophytes, and which have acquired the ability to digest keratin debris in the soil, have evolved to be able to parasitize animal’s keratin tissues [48].

Non-dermatophyte microorganisms can infect healthy skin, nails that have suffered some trauma and they can aggravate existing dermatophyte infections. They include *Candida* species, *Aspergillus* and *Alternaria* species. *C. albicans* infects especially fingernails when people’s hands are immersed in water regularly. Moreover, *C. albicans* differs from other strains because it penetrates the nail plate only after the infection of the soft tissue around the nail has spread. The majority of these fungal infections are localized, but they could cause an extensive necrosis of the involved tissue [49].

Onychomycosis can be classified into three classes: the first class is represented by Distal subungual onychomycosis (DSO) that is the most common form of onychomycosis. The fungus invades the nail bed and the lower part of the nail plate starting from the hyponychium. The infected organism then migrates through the underlying nail matrix, resulting in mild inflammation, focal parakeratosis and subungual hyperkeratosis, with two possible consequences: detachment of the nail plate from the nail bed and thickening of the sub-unitary region. In this way a subungual space is formed which can serve as a reservoir for a superinfection of bacteria and molds, giving the plaque a yellowish-brown appearance. DSO is generally caused by *T. rubrum* [50] . It could be develop both on fingernails and on toenails, although the second it is much more common.

Proximal subungual onychomycosis (PSO) constitutes the second class: it is a relatively uncommon subtype and it occurs when the disease agent invades the proximal nail fold through the cuticle area. The possible consequences of the infection are subungual hyperkeratosis, proximal onycholysis, leukonia and destruction of the proximal nail plate. *T. rubrum* is the main cause. PSO is very common in AIDS patients and it could be considered an early clinical marker of the infection [51].

Finally, there is White superficial onychomycosis (WSO): It is the least common form of onychomycosis and it occurs when the pathogenic agent invades the superficial layers of the nail plate. It can be recognized by the presence of well-defined "white islands" on the nail plate surface, which gradually widen as the disease progresses. As a result, the nail becomes soft, rough and friable. The inflammation is minimal, and the main causative agent of this type of infection is *Aspergillus niger*.

Fungal infection is often mistakenly considered to be a minor issue, a purely aesthetic problem that is not worth solving. However, onychomycosis has demonstrated to have a negative impact on patient social life, since patients are constantly afraid of being able to pass on the infection to their relatives. They may feel uncomfortable showing hands and feet, along with the strong impact that the infection may have on individuals at higher risk of exposure such as immunocompromised patients, where the infection stick more aggressively. Another issue about this pathology is the pharmacological

treatment that requires constant and long-term application, which can make the patient feel discouraged and stop the treatment [52]. Therefore, even if these infections are rarely life-threatening, they can have an important impact on public health and patients life [48].

Despite the high frequency of these infections within the population, the treatments option remain poor, and given the peculiarity of the affected area, new products are challenging to be assessed.

Treatment of onychomycosis has been a great challenge, failure rates and relapse rates have been high. Patient adherence, relapse due to reinfection, emergence of resistance strains and multiple organism infection may be the reasons hindering a successful onychomycosis treatment [53]. Current guidelines suggest oral or topical monotherapy antifungal treatment with terbinafine, amorolfine, itraconazole or cyclopirox [54]. The complete cure rate using oral or topical monotherapy remains in 30-50% [55].

Managing and controlling infected fungal nails, often depends on the site and severity of the infection, the causative organism as well as the patient's symptoms. According to the National Institute for Health and Care Excellence (NICE), if a patient has a dermatophyte or Candida nail infection, topical antifungal treatment can be used using amorolfine 5% lacquer, for people who have up to two affected nails or up to 50% involvement of the distal nail plate without nail matrix involved (NICE, 2019). This requires the patient to apply the topical antifungal lacquer once or twice weekly to the infected nail, after ensuring the nail is filed down short. Amorolfine 5% lacquer can be purchased over the counter. If the infection is on the fingernail, treatment should be continued for 6 months and for toenail infections, treatment is required for 9-12 months. Whilst on treatment, patients should avoid using cosmetic nail varnishes and artificial nails.

If self-care management alone and/or topical antifungal treatment is not successful, patients are advised to take oral antifungal treatment. Depending on the infection type, different antifungal drugs are prescribed. For dermatophyte nail infection, oral terbinafine is prescribed as first line treatment. Patients should take 250mg of terbinafine once a day for 6 weeks to 3 months for finger nail infection, and visible improvement would only be seen at the end of 2 months (NICE, 2019). For toenail infections, patients should take

250mg of terbinafine once a day for 3- 6 months, in which visible improvement may only be noticeable after 3 months of treatment. Oral itraconazole may be prescribed to patients as an alternative drug. It is prescribed as a pulse therapy where patients take 200mg of oral itraconazole twice a day for 1 week, followed by a drug free period of 21 days, after which itraconazole (200mg twice daily for a week) is taken (NICE, 2019). If Candida or non-dermatophyte nail infection is confirmed, oral itraconazole is prescribed as first line treatment, followed by oral terbinafine if an alternative drug is needed.

The use of combination therapy might be able to address this unmet clinical need in the future. Clinical advantage against monotherapy has already been reported in randomized controlled trial in the near past [56]. Combination therapy allows multiple drug targets to be blocked at the same time, and it can provide potential advantages such as faster fungicidal response and a smaller chance of drug induced fungal resistance [57]. Moreover, the use of a combination could limit dose-related side effects without compromising the clinical outcome. In vitro susceptibility test has shown some instance of synergism and additivism among antifungal drugs commonly used in onychomycosis treatment [58]. To accommodate the development of combination therapy, a rapid, reliable and reproducible method need to be established. Ideal assay methods should support both the susceptibility testing of monotherapy and combination therapy.

There are currently two standardized assays on susceptibility testing of antifungals outlined by the European Committee on Antimicrobial Testing (EUCAST) and Clinical Laboratory Standard Institute (CLSI). Both tests are derived from the broth microdilution method. However, there are still lack of evidence to suggest that it is the golden standard in testing antifungal susceptibility against dermatophyte fungus.

This method is labour-intensive and not readily applicable in routine laboratories. Agar-based methods, like disc diffusion assay, are attractive because of their simplicity and low cost, but they are not widely used for antifungal susceptibility testing. There are some studies which aim to find a correlation between broth microdilution method and disc diffusion assay and with clinical outcomes. However a general consensus is still missing,

suggesting the need for more correlation data to standardize the disc diffusion methods and broth microdilution for dermatophytes [59] [60].

In literature it is indeed easier to find references of studies concerning *C. albicans* and *Aspergillus* species to evaluate susceptibility to treatments, while the situation is more complex for dermatophytes. In fact, the results presented in previous studies of the in vitro susceptibility of dermatophytes to antifungals have shown considerable variations due to type of medium used, inoculum size, pH, incubation temperature, incubation period, and endpoint criteria [61] [62] [63] [64].

Besides onychomycoses, the high rate of mortality from systemic mould infections and the relatively limited efficacies of current agents have produced a significant interest in the use of antifungal combinations in these difficult-to-treat infections [65].

However, in vitro antifungal combination testing is controversial; tests are difficult to assess and the results depend on the methodology and analysis used [65].

The chequerboard dilution method and time–kill studies have become the most widely accepted techniques. In the classic checkerboard dilution scheme, all testing parameters remain the same, including medium, inoculum and incubation. The final result is the lowest concentration of drug A plus the lowest concentration of drug B in which the endpoint criteria are met. The MIC of each drug within the combination is expressed as a fraction of each drug alone. The fractions are then added to arrive at the fractional inhibitory concentration index (FICI) [66]. Synergism, indifference and antagonism are achieved when the FICI is ≤0.5, >0.5 to ≤4 and >4.

Moreover, recent works by W. J. MCAuley et al [65] and A. Baraldi et al [66] have examined the nail plate modification induced by onychomycoses and their relevant implications. These data supported the notion that infected nails presented a thicker but more porous barrier, and its eroded intracellular matrix rendered the tissue more permeable to topically applied chemicals, in particular when an aqueous vehicle was used. This findings should be taken into account when choosing an in vitro model to represent unhealthy nails.

The cosmetic industry

The cosmetics industry is an important sector in the Italian economy. In a survey carried out by the Cosmetica Italia study center and presented at the 2017 Cosmoprof in Bologna, it emerged that cosmetics are a growing sector. In fact, in 2016 it approximates 9,900 million euros in turnover with an increase of 0.5% over the previous year while the value of production reached 10,500 million euros. Also exports are significant, showing an increase of more than 12%, for a value close to 4,300 million euro [67].

The worldwide trend of some important categories, such as nail polish and hair removal products, shows strong growth in the first category (average annual variation of + 46%) and a more moderate one in the second category (average annual variation of + 3%)). The 2014-2015 water-based nail polish boom was then waning due to the lack of technical qualities of the products. Only recently water-based products have been developed with good quality characteristics reflecting their claims. Regarding the most successful nail polish claims, the "long-lasting" functional claim dominates the scene (31% of the total launches in 2015), with the claim "seasonal" (21%) and “ethical” (16%). Moreover, “Easy to use” and drying time related claims are quite popular [68].

Nail cosmetics

Cosmetics products intended for use on nails could have both decorative and protective functions. In the event of mycotic infections cosmetics products could promote nail regrowth, even if they would not be a replacement for pharmacological therapy. In this field, it is possible to find several kind of products, from hydro alcoholic based products with film-forming properties, with antimycotic agents or natural ingredients able to disadvantage fungal proliferation. These products could have a cover function, hiding a nail affected by a specific pathology which usually has a strong impact on the aesthetics. In case of weak and dystrophic nails for example, a selection of strengthening products is available on the market, while for anomalies of the nail plate shape it is even possible to appeal to artificial nails. While this could sounds like an innovative practice, it is actually in use in China since 600 a.C. under Chou dynasty where artificial nails where made by silver, gold and precious stone. Of course these cosmetics corrections are temporary, and they are not

intended to provide a cure, but exclusively cover and camouflage of the undesirable characteristics [69] [19].

Among the products most widely used there will be:

Nail polish, realized in the past with eggs, flowers and wax both in the ancient Egypt and China. The first modern nail polish as we know it has been formulated in 1920 by the Charles Revson Company, currently know as Revlon. It was inspired by enamel used to color motors vehicles [70].

The first plishes were made by pigmented nitrocellulose dissolved in organic solvents which by evaporating let a glossy and hard film above the surface of the nails. Afterwards, nitrocellulose has been replaced with polimers in order to enhance flexibility and resistance of the film [69].

The most widely used ingredients include:

- Resins: two main kind of resins in different concentrations are employed for nail polish formulations based on the desired effects. For base coat formulas, made with the aim of improving nail polish adhesion to the lamina, have a higher amounts of soft and flexible resins, while top coat formulas, made to enhance polish gloss and durability, are made with high percentage of glossy and hard resins. From a chemical point of view the most common are nitrocellulose, methacrylate polymers and vinyl polymers. Nitrocellulose is widely used since it creates a shiny and hard film, permeable to oxygen, which is a critical property for human nails health.
- Solvents: their own purpose is to dissolve or suspend the pigments. Usually used in combinations, they need to evaporate quickly after application of the product, allowing the polish to dry as soon as possible. The most commonly used are ethyl acetate, n-butyl acetate and isopropyl alcohol.
- Plasticizers: added to improve resistance and flexibility of the film.
- Pigments: oxides and mica are responsible for the final appearance of the polish. In Europe, organic colorants need to be included in regulation 1223/2009 in order to be used in cosmetic formulations, while inorganic pigments need to be compliant with heavy metal residuals [71].

- Tixotropic agents such as hectorite and bentonite.
- Color stabilizers.

Nail polish could expert also a protective function, limiting the contact of the nails with water and detergents, slowing down transonychial water loss and increasing the hydration of the nail plate [72].
However, regular and prolonged use of nail products could also lead to nail fragility [70].

Moisturizing products: they are usually cream and lotion formulations containing occlusive ingredients, such as petrolatum derivatives or lanoline, humectant ingredients, such as glycerine or propylene glycol and other components like proteins, silica, alfa-hydroxide acid and urea to improve water retention capacity of the lamina. These products exert their activity at best when used in occlusion overnight by the use of a cotton gloves [73].

Cuticles products: usually lipogel formulations desiged to soften the cuticles in order to make them easily removable.

Nail polish removers: prodcuts desigend to remove nail polish from the nails. On the market, it is possible to find the well-known acetone based products, which used could lead to nail damage and in predisposed individuals even cause dermatitis and irritation, or acetone free removers, made with ethyl acetate, butyl acetate or ethyl lactate. It is now possible to find also disposable patches soaked with gamma-butyrolactone [70].

Nail Gel: nail gel application is based on three chemical-physical principles: adhesion, polymerization and evaporation. The first process consist in the adhesion of the product on the surface of the nail, which need to be clean and oil-free as possible to have the best performance. Usually the product is just a solution of single monomers. Polymerization process is triggered by the presence of a promoter or by an energy source, such as an UV light lamp, which induces formation of cross-bonding within monomers and the creation of a hard film. Polymerization causes a shrinkage of the material up to 20% of

the mass and could sometimes induces heat release. With evaporation of the solvents the structure became rigid [69].
This technique is wide-spread nowadays, however it takes some challenging concerns with it: aggressive remotion of the gel could lead to nail damage, while the photo sensibility is a tangible risk in patients assuming oral therapies such as tetracycline [71]. Moreover the continue use could lead to contact dermatitis and enhance onychomycosis susceptibility.

The EU Regulation 1223/2009 (Art.10 – Safety Assessment) states [74]:

"In order to demonstrate that a cosmetic product complies with Article 3, the responsible person shall, prior to placing a cosmetic product on the market, ensure that the cosmetic product has undergone a safety assessment on the basis of the relevant information and that a cosmetic product safety report is set up in accordance with Annex I. (…) The Commission, in close cooperation with all stakeholders, shall adopt appropriate guidelines to enable undertakings, in particular small and medium-sized enterprises, to comply with the requirements laid down in Annex I. Those guidelines shall be adopted in accordance with the regulatory procedure referred to in Article 32(2)."

However when talking about nail care products, we found ourselves in the "grey area" of the guidelines, since there is not an harmonized document referring to for specific quality assessment for this kind of cosmetics.
Harleen Arora and Antonella Tosti in their review paper "Safety and Efficacy of Nail Products" published on Cosmetics in 2017 summarized the results of the data available regarding common nail products and their safety and efficacy [75]. They concluded that although there are efforts in place by government agencies both in EU and US to increase safety on cosmetics, legislation will be inadequate until more hard evidence about the safety of nail cosmetics is generated.
This is even more evident when talking about efficacy assessment. In fact, while searching the literature on the matter it is immediately clear how poor it is.

AIM OF THE PROJECT

The literature on nail products is vast as it offers several experimental works and procedures to assess novel actives and products permeation through the nail barrier. Nonetheless, a general consensus on the protocols has not been reached yet. For this reason, finding scientific references of standardized procedures meant to evaluate and characterize nail products is no easy task. It becomes even more challenging when it comes to the assessment of products designed to act on particular nail issues and diseases, such as those involving mechanical properties, which could have impact in term of both efficacy and safety.

Therefore, the first aim of this three years' work has been the development of an in vitro model for nail, which is suitable to become the gold standard to objectively support products commercial claims, but also a tool in safety evaluation process.

Bovine hooves membranes are well-known for their use as human substitutes for studying nail permeability and effects of ingredients on the nail. For this reason, the first part of the work has been devoted to production and characterization paradigms development and thorough description. In particular, mechanical characterization has been carried out using a novel technique already applied in vivo, along with the characterization with several surface analysis techniques. This has been done in order to understand which properties result more relevant within the selection of the model for a specific in vitro study, but also to assess the adaptability to a wide range of techniques.

Subsequently, the model has been applied to the development of protocols to evaluate products in vitro. In the cosmetics field, the focus of the work has been the evaluation of several products meant to improve nail conditions affecting mechanical properties, such as brittle nail syndrome.

In terms of pharmaceuticals application, onychomycoses has been chosen as the target of interest, since it is a wide diffused pathology, the treatment of which is still often not successful.

Each of the studies reported in this experimental work addresses a different step in the efficacy assessment of a product designed to be used for onychomycoses prevention or treatment, in an area where the assessment protocols are scarces and not yet standardized.

CHAPTER 1

IN VITRO MODEL PRODUCTION AND CHARACTERIZATION

INTRODUCTION

Several studies can be found using bovine hoof membranes as a nail in vitro model. Most of them are actually permeability studies, while it is very hard to find reference in literature related to the use of those membranes to evaluate safety and efficacy of products. In facts, some effects, both desirable or not, are not strongly related to the penetration of the products through all of the nail plate keratin layers. There are morphological characteristics and properties such as mechanical ones which could be affected even by a product acting on the surface of the nail.

Moreover, hoof membranes production is not a standardized procedure, as well as membranes characterization which is a critical step to deal with when we are looking for a specific in vitro model.

In this chapter, hoof membranes production is described in detail, along with the characterization techniques which could be applicable and useful for studying the model, in order to define the parameters of selection of the membranes for specific purposes as well as parameters to be used as safety and efficacy studies endpoint.

MATHERIALS AND METHODS

Membranes Production

All hoof membranes have been obtained from fresh slaughtered 3 years old cattle (Azienda Agricola Pluderi Marcellino, via Villa Serafina 19, San Colombano al Lambro).

Freshly slaughtered bovine hooves were dipped in liquid nitrogen, cored according to the chosen diameter by using a Plug cutter Rolson® with 16 mm diameter, Rolson Tools LTD (Twyford, United Kingdom), and cut in slices with

a precision lathe Graziano SAG 12 (Tortona, Italia), equipped with HSS blade (high-speed-steel), Freud® S.p.A. (Tavagnacco, Italia) and an analog Centesimal Comparator with magnetic support Borletti®, LTF S.p.A. (Antegnate, Bergamo). Membranes are codified as follow: x.y.z, where x is the number or the code referring to the hoof, y is the number of the carrot and z the number of the membrane in ascending order form the most superficial one to the deepest one.

Figure 3. Hoof number 4 as an example of carrots numeration.

Before every experiment and evaluation the membranes were sanitized with a sequence of washing with Ethanol 70% v/v and a Benzalkonium Chloride mixture [0,4 g Benzalkonium Chloride (Farmalabor); 70 g isopropyl alcohol (Carlo Erba); distilled water to 100g] and then maintained in a climatic chamber at 25°C and 40% R.H (ClimaCell 111 MMM).

Furthermore, in order to modify membranes porosity, some of them were subjected to a compression machine. The membrane morphology analysis was performed by a digital microscope model BW 1008 to evaluate the surface desquamation features and the thickness measurement has been performed using the Nail StrainStress Meter NM 100, COURAGE&KHAZAKA electronic GmbH (Cologne, Germany) described in details later in the “Mechanical Properties” section.

Membranes Characterization

Superficial properties

SEM and elemental composition analysis

Scanning electron microscopes use electrons rather than light to form an image. Electrons have a much smaller wavelength than light, so the SEM can resolve much smaller structures than the traditional light microscope. The smallest wavelength of visible light is approximately 400 nm (400 millionths of a meter), whereas the wavelength of electrons used in the SEM is approximately 0.005 nm (5 trillionths of a meter). There are many advantages to using the SEM for medical research instead of a light microscope. The SEM can produce three-dimensional (3-D) images with a large depth of field, which allows a large amount of the sample to be in focus at one time. The SEM also produces images of high resolution, which means that closely spaced features can be examined at high magnification (up to ×200,000) and that structures as small as 2.0 nm can be seen. The ability to produce 3-D medical images with a combination of a larger depth of field, greater resolution, and higher magnification than light microscopes makes the SEM an important diagnostic tool in medical research [76]. As reported by Bercker et al [22] scanning electron microscopy helps clarify basic nail plate structure. Moreover, in literature there are several examples of use of this technique to study and characterized pathological nails over years [77] [78] [79].

For this work, has been used a Phenom XL Scanning Electron Microscope (Phenom World, part of Thermo Fisher Scientific). Moreover, for the elemental characterization of the samples EDS (Energy Dispersive Spectroscopy) technology embedded in the microscope system has been used. In practice, backscattered electron images in the SEM display compositional contrast that results from different atomic number elements and their distribution. EDS allows us to identify what those particular elements are and their relative proportions. EDS is a technique that analyses X-rays generated by the bombardment of the sample by an electron beam. X-ray detector and control software are combined in one package. This Elemental Identification (EID) software package allows the user to program multiple point analysis, and identify any hidden elements within the sample.

Bovine hooves membranes and human nails selected for the analysis are listed below (Table 1).

Table 1. Code and specifications of the membranes selected for the SEM analysis.

Code	***Specifications***
J.8.1	Superficial membrane; Dark
K.2.1 (section)	Pressed membrane
K.2.4	Pressed membrane; Medium dark
J.2.7	Pressed membrane; Light
J.6.11 (section)	Pressed membrane
U-M	Human nail (freshly cut)
U-A	Human nail

The sample was placed on the stub and loaded in the standard sample holder. The images are taken at different magnification and locations using an acceleration voltage of 15kV. The EDS analysis were performed on different spots or regions, using an acceleration voltage of 15kV for great analysis results.

Immunostaining and confocal laser scanning microscopy pilot study

Confocal microscopy, developed and patented by Marvin Minsky in 1955, uses optical imaging to create a virtual slice or plane, many micrometres deep, within the tissue. It provides very-high-quality images with fine detail and more contrast than conventional microscopy. In addition, the imaging technique allows for reconstruction of virtual 3-dimensional (3-D) images of the tissue when multiple sections are combined.

In literature it is possible to find many examples of use of this techniques on nails in vivo [80] but nothing on its use on nails and/or hooves membranes in vitro/ex vivo.

Bovine hooves membranes and human nails selected for the analysis are listed below (Table 2):

Table 2. Code and specifications of the hoof membranes selected for the assay

Code	Specifications
J.8.1	Superficial
J.6.9	Deep
K.2.4	Pressed/Superficial
J.6.11 (section)	Pressed/Deep
326 (I5081)	Superficial

Table 3. Code and specifications of nail clippings selected for the assay.

Code	Specifications
A	25 years old subject
P	45 years old subject (freshly cut)
C	11 years old subject (freshly cut)

All nail samples were fixed with 70% ethanol.

Nails were washed twice for 30 min in PBS and incubated for different times (24h, 72h, 10 days) in the PTA blocking solution (1% w/v BSA and 0.02% w/v Tween 20 in PBS Tween Albumin).

Immunostaining was performed by incubating the sample with primary monoclonal antibody to pan-cytokeratin (clone LU5, Bachem, UK) diluted 1:100 in PTA, for 1h at room temperature and then at +4°C overnight. After two washes with PTA (10 minutes each), samples were incubated for 30 min with a secondary antimouse antibody (fluorescein isothiocyanate (FITC)-conjugated antibody, Sigma-Aldrich, Saint Louis, MO, USA) diluted 1:100. Nuclear staining of deoxyribonucleic acid (DNA) with Hoechst 33258 dye (0.5 µg /mL, Sigma-Aldrich, Saint Louis, MO, USA) was also performed on every sample. The fluorescence images were obtained using a Confocal Laser Scanning Microscope (Leica, TCS SP5II, Leica Microsystems, Milano, Italy) by acquiring green and red fluorescence signals at 0.3-mm intervals. Image analysis was performed using the Leica Confocal Software. Microscope parameters were kept constant for all the analysis.

Moreover, samples treated with PTA only were used as a blank and samples incubated with the secondary antimouse antibody only were used to rule out the case of an aspecific staining.

Zeta Potential and Isoelectric Point

The zeta potential and isoelectric point were determined using SurPASS ™ 3 (Anton Paar, Austria).

SurPASS™ 3 is a high-end electrokinetic analyzer featuring fully automated zeta potential analysis of macroscopic solids in real-life conditions. SurPASS™ 3 employs the classic streaming potential and streaming current method for a direct analysis of the surface zeta potential. The zeta potential is related to the surface charge at a solid/liquid interface and is a key parameter for understanding surface properties. Automatic pH scans and time-dependent recording of adsorption kinetics allow for a deep understanding of the surface chemistry. The elaborate measuring cells of the instrument suit various sample geometry, size or origin.

A background electrolyte of 1 mM KCl solution was used and the pH was adjusted in the range of 2.5–9.5 with 0.1 M HCl and 0.1 M NaOH. Nitrogen supply has been used during all the analysis to prevent carbon dioxide dissolution and help maintain the parameters stable during all the analysis. The zeta potential is calculated automatically by the instrument software using the Smoluchowski equation (Figure 4) which describe the dependence of the streaming current (ΔI_{str}) or streaming potential (ΔU_{str}) on the pressure (ΔP) applied on an aqueous electrolyte flowing through a channel. η is the dynamic viscosity of the fluid, ε_0 and ε_r are the vacuum permittivity and the relative permittivity, respectively, L is the channel length, A – its cross-section, K_B is the specific electrical conductivity of the channel.

$$\zeta_I = \frac{\eta}{\varepsilon_0 \varepsilon_r} \frac{L}{A} \frac{\Delta I_{str}}{\Delta P}$$

$$\zeta_U = \frac{\eta}{\varepsilon_0 \varepsilon_r} K_B \frac{\Delta U_{str}}{\Delta P}$$

Figure 4. Equation showing the relation between streaming current/streaming potential and zeta potential.

The cell and tubing were carefully rinsed with Millipore water prior to each measurement and the flow checked.

Several tests were needed to identify and choose the most adequate measuring cell for a specific sample such as nails and hoof membranes.
The cylindrical cell is mainly used for the investigation of natural or technical fibers and fabrics, granular samples and coarse particles: we selected this cell for human nail clippings analysis, managing the sample as a granular one.
The standard clamping cell (10x20 mm) is the tool of choice for measuring planar surfaces like polymer films and sheets, metals, ceramics, glass or semiconductor wafers and it seemed to suit hooves membranes analysis. However, it was necessary to mount two membranes together to cover the entire surface of the sample holder.
An extra assay was performed using a customized clamping cell (10x10mm).

Contact Angle

The main index used to evaluate the wettability of a solid surface is contact angle (CA). The phenomena of a surface wetting occurs when a liquid contacts a solid, and the liquid layer spreads on the solid surface [81]. Different materials have different wetting properties due to the intermolecular interactions between the liquid and the solid [82]. Thomas Young was the first to mathematically describe the CA in a work dated 1805 [83]. Conventionally, CA describes the behaviour of a liquid droplet on a solid surface in air, and it is defined as the internal angle between the tangent at the intersection point air-liquid-solid and the solid surface. Generally, when the liquid considered for the assay is water, solid surfaces with CAs < 90° are considered hydrophilic, while surfaces with CAs > 90° are considered hydrophobic. However, recent studies have determined that the value to be used as a limit between hydrophilicity and hydrophobicity for a smooth solid surface actually is a CA of 65° [84] [85].

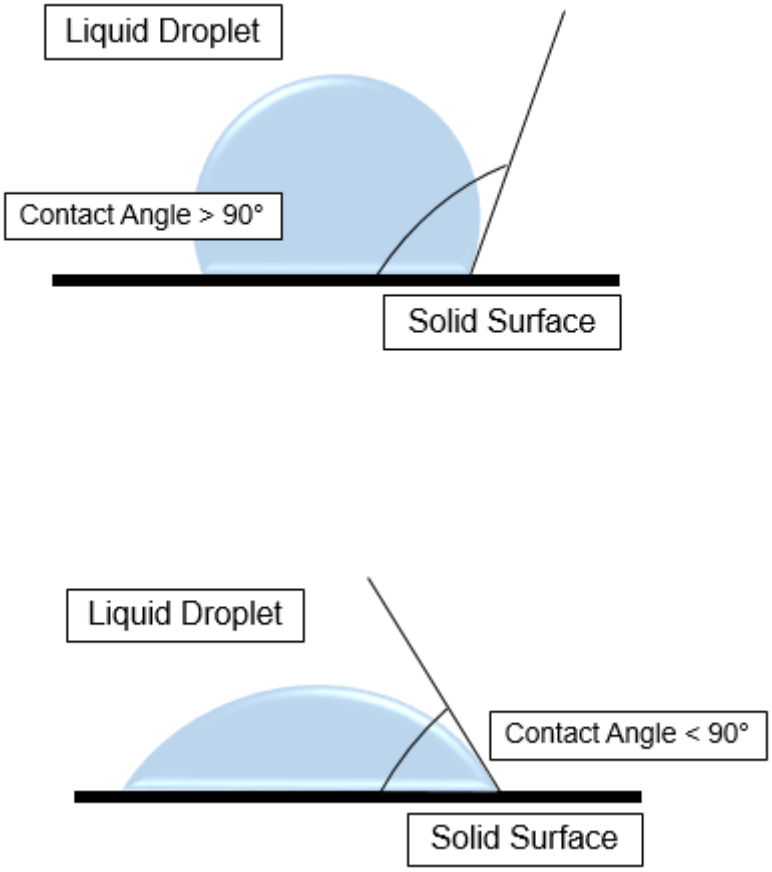

Figure 5. Contact angle schematic representation.

In literature is reported the use of contact angle measurement to predict cosmetic adhesion of cosmetic products and ingredients on skin [7]. Since nails could be the target for the application of cosmetics and pharmaceutical products, studying their superficial properties could be crucial in design and evaluation of new formulations. Therefore, contact angle measurements could be an essential data for characterization of the in vitro method chosen for product testing.

In this work, a group of membranes with different properties was characterized using Contact Angle Meter DMe-211Plus (KYOWA) using 10µL as standard volume for the chosen liquid. In this case the characterization have been performed using MilliQ® water.

Figure 6. Contact Angle Meter DMe-211Plus (KYOWA) from KYOWA website

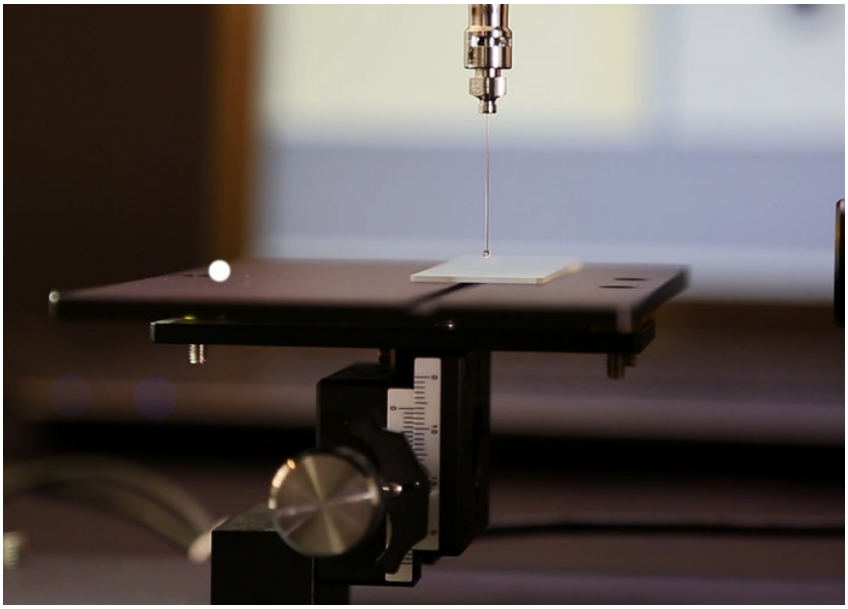

Figure 7. Contact Angle Meter DMe-211Plus (KYOWA) sample stage from KYOWA website

22 flaky or flaky/smooth membranes in thickness range of ~300μm have been analyzed while mounted on the custom made PTFE support, while 61 thinner membranes (~200 μm thickness range) have been analyzed as a solid surface, just positioned on the sample support of the instrument. As for the other assays, the membranes were left drying for at least 3 days after the last

cleaning/sanification process inside the climatic chamber at 25°C and 40% R.H (ClimaCell 111 MMM).

Different methods for the classification of membranes wettability have been chosen including not only the absolute contact angle value itself, considering CA < 65° wettable/hydrophilic and CA > 65° poorly wettable/hydrophobic, but also the evaluation of the contact angle percentage variation over time (from the deposition time of the drop 0s to 60s).

Statistical analyses was carried out on the results using a T-Test. A significance level of 5% was chosen, so the changes were considered statistically significant for $p < 0.05$. Other conventional symbols used are: not significant (n.s.) for $p > 0.05$; slightly significant (*) for $p \leq 0.05$; significant (**) for $p \leq 0.01$; very significant (***) for $p \leq 0.001$.

Mechanical Properties

Mechanical characterization were carried out using Nail StrainStress Meter NM 100, COURAGE&KHAZAKA electronic GmbH (Cologne, Germany). The Nail StrainStress Meter NM100 was developed and patented by our research group in collaboration with Courage & Khazaka [86]. The aim of the instrument is to allow the quantitative determination of relevant mechanical properties of living and reconstructed tissue, of skin appendages and polymeric structures in association with the tissues. The equipment works in-vivo and in situ and in non-invasive way [86].

Figure 8. Nail StrainStress Meter NM 100

As described by Perugini et al., the instrument consists of a load cell, which moves with the head of compression which is able to go down with high precision subjecting the nail at a force varying from 0.2 to 8 N, since the device features a high precision force dosimeter. The three sets of head and related support available allow to perform measurements of direct compression, transversal deflection and longitudinal deformation of the nail plate (Figure 9).

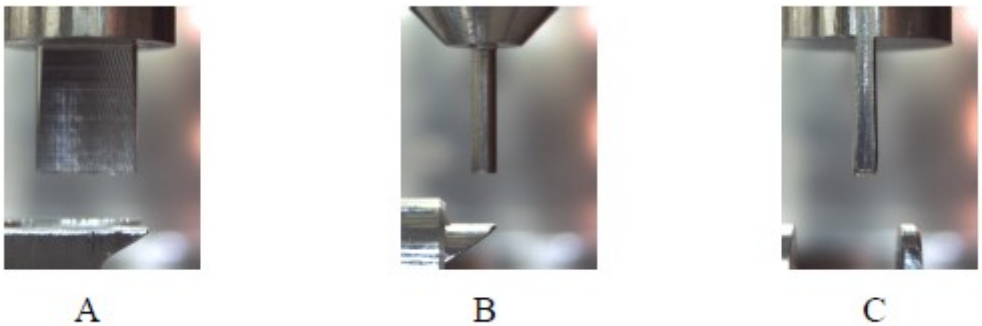

Figure 9. Nail StrainStress Meter NM 100 available sets: A) Set 1 to measure transversal deformation; B) Set 2 to measure compression; C) Set 3, to measure longitudinal deformation.

The heads can be driven automatically in smallest steps (as small as 0.1 μm) down on the sample. During the deflection the force is constantly monitored. As soon as the head meets the sample positioned on the support, the force needed increases. The results are curves displacement versus applied force. From these curves linear regression lines are built up and from their slopes interesting mechanical nail parameters are obtained. The results are analysed in real time by the software Courage+ Khazaka electronic GmbH and then exported to excel to further analysis.

In particular, the assessment of the resistance to compression with set 2, expressed as the Firmness Index (FI), measures the cohesion of the nail matrix and indicates directly the structural strength of the sample, while set 1 allows the assessment of the transversal deformation resistance of the sample, expressed as the Viscoelasticity Index (VI).

The thickness of the nail is easily and with extreme accuracy determined by the compression analysis using the difference between the total run of the head to touch the support and the first point of contact with the sample.

As the described in the paper, the system has been validated to be used for in vivo assessment, and in this work the assay has been applied for the characterization of hoof membranes. At the moment, a protocol has been developed for the mechanical characterization of hoof membranes produced with a thickness ~300μm, which are in fact the closest model to the human nails.

All mechanical properties measurements were made in an air conditioned room with controlled temperature and humidity (T 25°C, R.H. 50 ± 5%) on cleaned and dried membranes which have been stored inside the climatic chamber at 25°C and 40% R.H (ClimaCell 111 MMM).

Moreover, in order to have membranes to be dried in a valid position for the mechanical assessment, which could mimic a real-life in vivo situation as close as possible, the samples were mounted on custom made PTFE support as reported in figure 10.

Figure 10. Hoof membranes mounted on PTFE custom made supports.

In order to minimize the possible influence of the length of the membrane on the analysis, the position on the support has been standardized to 5 mm as reported in the figures below.

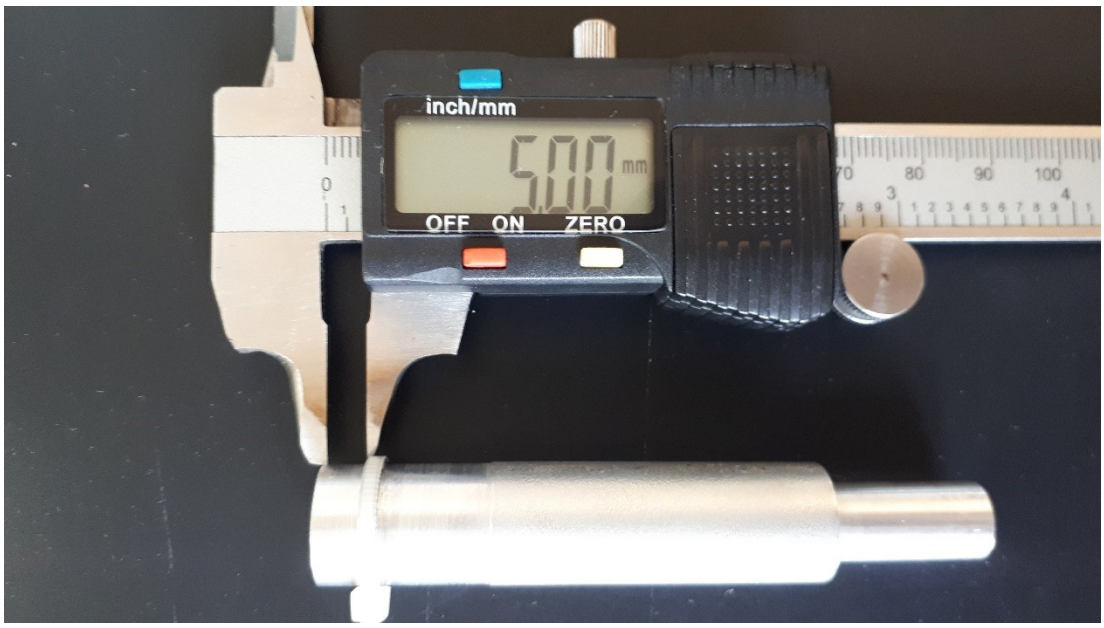

Figure 11. Length standard

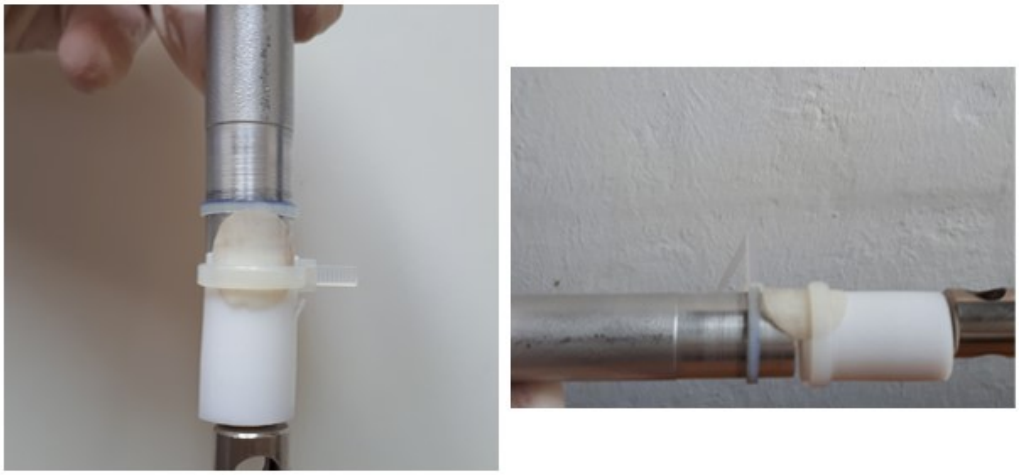

Figure 12. Membrane length standardization

As reported by Perugini et al., using smaller force intervals lead to a lower variability within the results. Therefore, the instrument settings chosen for membranes mechanical properties measurements are reported in the table below:

Table 4. StrainStress Meter NM100 settings for mechanical parameters measurements of membranes

SET	**Step size (mm)**	**Force (N)**	**Parameter**
Set 1	0,005	1,5	Viscoelasticity Index (VI)
Set 2	0,001	2	Firmness Index (FI) and Thickness

Results of each mechanical parameter (VI, FI and Thickness) are expressed as mean result of at least 3 measurements on each membrane. The total mean is calculated as the arithmetical mean of all the mean value for each membrane.

Reproducibility Test and Water Effect

Before starting the data collection, a reproducibility test has been performed: 20 membranes of about 300µm have been classified and selected for the test and VI, FI and Thickness were measured at three different time points T0, T1 (24 hours after T0) and T2 (1 week after T0). The mean percentage variation of the parameters value over time and the standard deviation among the three

values have been calculated in order to evaluate the reproducibility of the measure.

A second test has been performed with the aim to assess the water effect on membranes and so the membranes appropriate drying-time before mechanical assessment. For this study, 20 membranes of about 300μm have been classified and selected. Mechanical parameter have been measured at the beginning (T0), after 10 minutes soaking in 37°C water (T1), after 24 hours (T2), after 72 hours (T3) and after 1 week (T4). The mean percentage variation of the parameters value over time and the standard deviation among the values have been calculated.

Correlation Analysis

Then, a statistical analysis on the data collected from 265 hoof membranes and human volunteers (9 subjects; 37 values; position 1; 5; 6; 10 were excluded) was performed. The Pearson correlation coefficient is a statistic that measures linear correlation between two variables and it has been chosen to give an estimate of the correlation relationship among the variables VI, FI and Thickness [87]. It has a value between +1 and −1, where 1 is total positive linear correlation, 0 is no linear correlation, and −1 is total negative linear correlation. The correlation reflects the noisiness and direction of a linear relationship, but not the slope of that relationship, nor many aspects of nonlinear relationships. Moreover, to reduce the influence of outliers, which have been demonstrated to have an impact on Pearson's correlation, Spearman's Rank correlation values as been calculated as well for comparison [88]. There is no general consensus on the how to name the strength of the relationship for different coefficients [88] [89]. Based on literature I decided to apply the criteria used by Chan et al. [90] as reported in table below.

Table 5. Strength interpretation of correlation coefficient

Correlation Coefficient		
Direct	Inverse	Strength Interpretation
1	-1	Perfect
0,9	-0,9	Very Strong
0,8	-0,8	Very Strong
0,7	-0,7	Moderate
0,6	-0,6	Moderate
0,5	-0,5	Fair
0,4	-0,4	Fair
0,3	-0,3	Fair
0,2	-0,2	Poor
0,1	-0,1	Poor
0	0	None

Correlation between observed variables such as VI and Fi and fixed values such as membranes position within the carrot is evaluated through the use of linear regression method.

Moreover, in vivo measurements have been performed as a comparison, using the same settings as for the in vitro assay (Table 4). All in vivo data reported have been obtained from volunteers female subjects aged 14-30 following the requirements highlighted by Perugini et al. [86]:

- Clean and dry nail plate, with no use of nail products or treatments for at least 3 days before the assay;
- Pre conditioning of the volunteer at least 15 minutes before the assay;
- Assessment of the morphological characteristics of the nails.

Fingers have been conventionally numbered from 1 (left pinky finger) to 10 (right pinky finger). Nail length of the volunteers was measured in three different points L1, L2 and L3 (Figure 13) through a digital software CoolinTech 2.0 after calibration with a known value through a calibre.

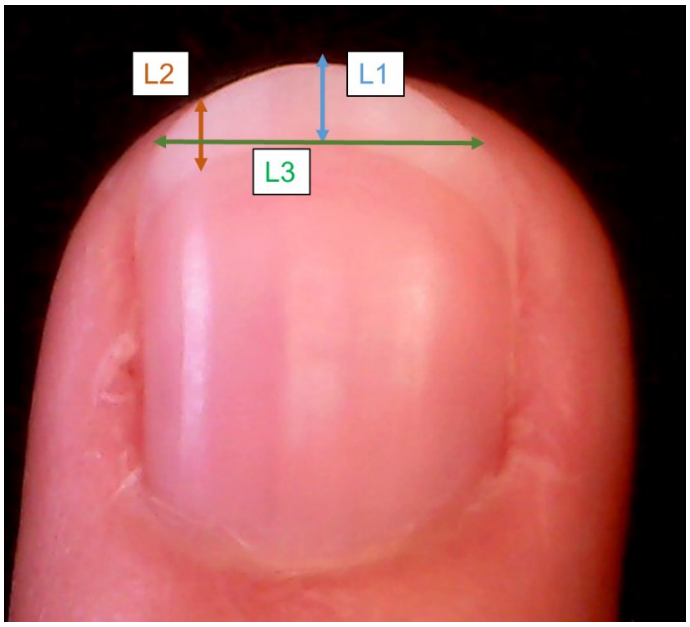

Figure 13. Schematic picture of the measure taken on the human nail with the Software CoolinTech 2.0

RESULTS

Membrane Production

With the production method described above more than 400 membranes have been produced and analysed, within two thickness range: membranes with a thickness of about 200µm designed for permeability studies and membranes with a thickness of about 300 µm to be used to simulate in vivo response in studies concerning efficacy and safety of ingredients and final products.

After the sanification procedure, for all the membranes the thickness range was checked with a calibre. Then, all the membranes were photographed with a digital microscope in order to be classified according to their morphological properties in two main groups: smooth (S), flaky (F) and Flaky/Smooth (F/S) when in the middle of the two type.

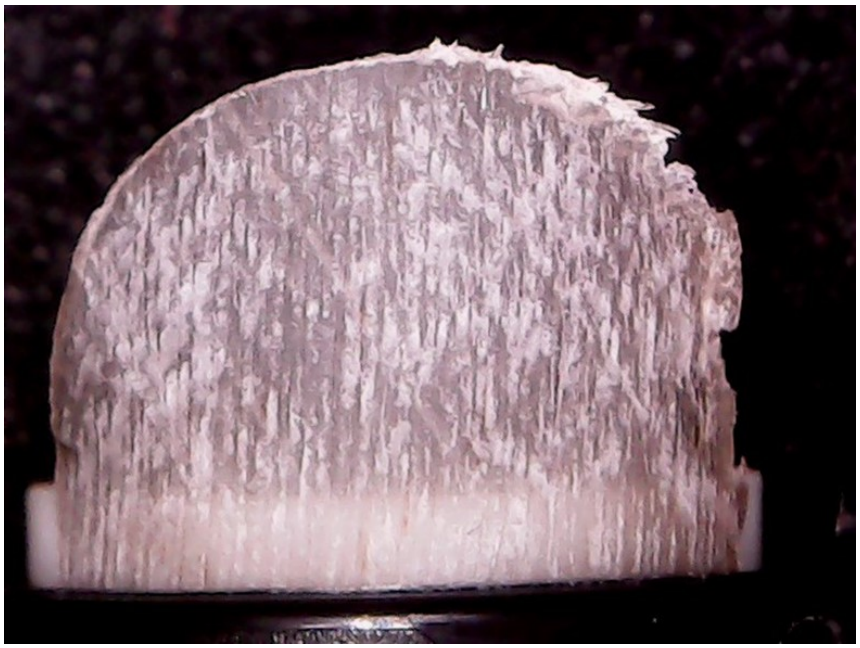

Figure 14. Example of flaky membrane

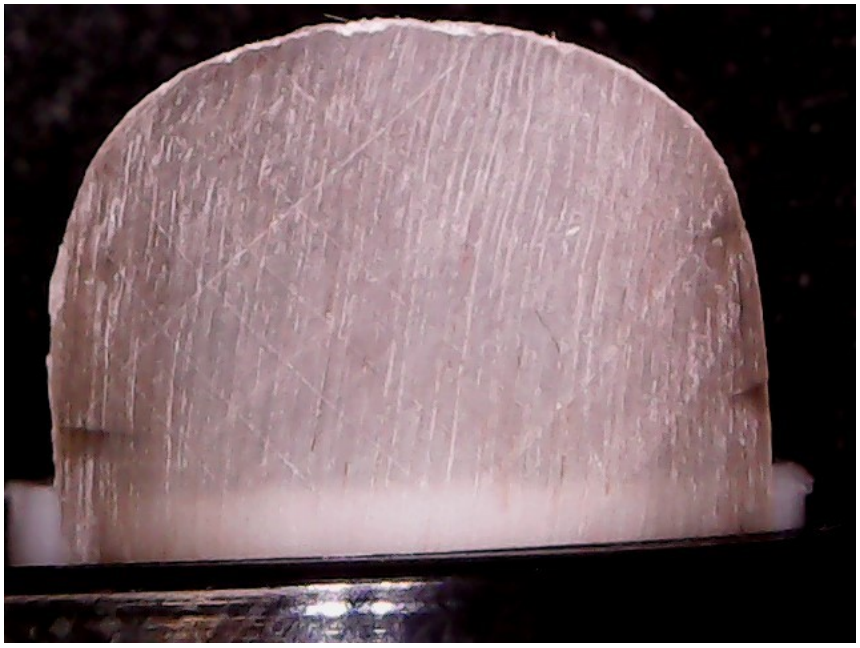

Figure 15. Example of smooth membrane

This classification method based on morphological properties is the first step in choosing the adequate model for product testing.

Here below are reported the results obtained for 74 hoof membranes in the thickness range around 200 μm. Thickness measures are taken with a calibre in at least 3 different point of the sample (Thickness MIN: is the lowest value registered; Thickness MAX: is the highest value registered for that sample).

Table 6. Hoof membranes (thickness range ~200µm) classification

Code	Morphology	Thickness MIN (µm)	Thickness MAX (µm)	Difference (µm)
A.6.1	F	230	280	50
A.6.2	F	130	200	70
A.6.3	F	150	230	80
A.6.4	F	220	290	70
A.6.5	F	240	300	60
A.6.6	F	110	120	10
A.6.7	F	140	370	230
A.6.8	F	180	230	50
A.6.9	F	150	170	20
A.6.10	F	180	300	120
A.6.11	F	180	270	90
A.6.12	F/S	140	200	60
A.6.13	F/S	160	270	110
A.6.14	F/S	170	260	90
A.6.15	F	180	260	80
A.6.16	F/S	130	200	70
A.6.17	F/S	140	170	30
A.6.18	F/S	140	230	90
A.6.19	F/S	150	220	70
A.6.20	F/S	190	300	110
A.6.21	F/S	190	200	10
B.1.1	F	160	300	140
B.1.3	F	180	240	60
B.1.7	F	130	130	0
B.1.8	F	190	200	10
B.1.9	F	150	170	20
B.1.10	F	130	200	70
B.1.11	F	130	150	20
B.1.12	F/S	130	200	70
B.1.14	F	170	230	60
B.1.15	F	170	250	80
B.1.16	F/S	120	180	60
B.1.17	F/S	230	330	100
B.1.19	F	160	170	10
A.1.1	F	220	250	30
A.1.B	F	190	210	20
A.1.1	F	250	280	30
A.1.2	F	200	210	10
A.1.3	F	190	200	10
A.1.4	F	200	210	10

A.1.5	F	270	300	30
A.1.6	F	200	225	25
A.1.7	F	270	280	10
A.1.8	F	230	270	40
A.1.9	F	210	220	10
A.1.10	F	220	250	30
A.1.11	F	180	230	50
A.1.12	F	150	180	30
A.1.13	F	170	210	40
A.1.14	F	190	200	10
A.1.15	F	180	240	60
A.1.16	F	210	230	20
A.1.17	F	220	240	20
A.1.18	F	130	220	90
A.1.19	F	270	300	30
A.1.20	F	250	280	30
A.9.2	F	200	210	10
A.9.3	F	160	230	70
A.9.4	F	270	280	10
A.9.5	F	230	260	30
A.9.6	F	190	210	20
A.9.7	F	210	310	100
A.9.8	F	220	240	20
A.9.9	F	220	280	60
A.9.12	F/S	220	240	20
A.9.13	F/S	180	190	10
A.9.14	F/S	240	270	30
A.9.15	F/S	190	230	40
A.9.16	F/S	190	220	30
A.9.17	F/S	220	250	30
A.9.18	F/S	220	250	30
A.9.19	F/S	190	240	50
A.9.20	F/S	220	240	20
A.9.21	F/S	220	250	30
Mean		**188,4**	**235,9**	**47,5**
SD		**39,9**	**45,9**	
%SD		**21,2**	**19,5**	

The membranes obtained in this thickness range have been all classified as flaky or flaky/smooth. The mean lowest and highest thickness values are respectively 188,4 μm and 235,9 μm with a mean percentage standard deviation of 20,35% and a mean difference between the two extreme values

of 47,5 µm, which we considered acceptable. In order to guarantee the best reproducibility as possible, membranes with the higher variability between the highest and the lowest thickness value must be discharged and will not be used in experiments.

Then, 265 membranes both smooth and flaky in the thickness range around 300 µm were mounted to the PTFE support to undergo a deeper statistical analysis of the thickness values through the use of the Nail StrainStress Meter NM 100. This thickness range is closer to the human nail thickness values. Therefore, it has been decided to apply the Nail StrainStress Meter for a precise analysis of the thickness values distribution

.

Figure 16. A membrane in the thickness range of 300-400 µm mounted on a PTFE custom made support

All the 265 membranes mounted were analysed with Nail StrainStress meter set 2 to obtain precise thickness values. The thickness value of each membrane is expressed as the mean value of at least 3 measurements in 3 different points of the sample surface. No values have been left out for this analysis.

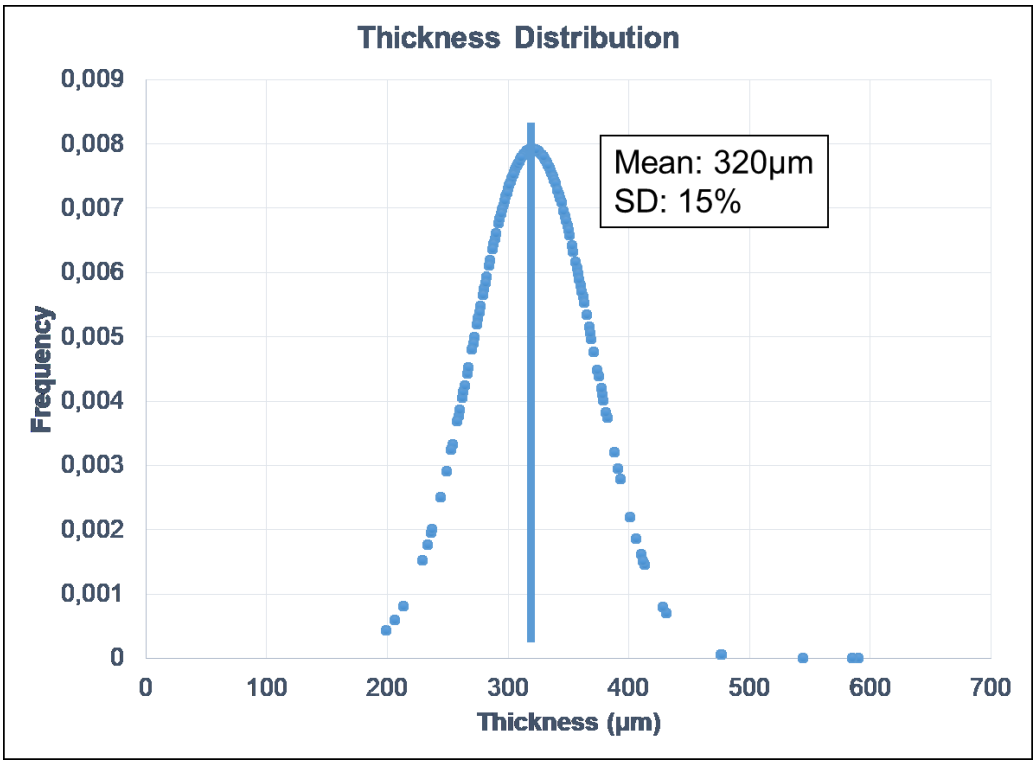

Figure 17. Hoof membranes thickness values distribution

As shown in Figure 17, the thickness values obtained are close to a normal distribution around the mean value of 320µm with a percentage standard deviation of 15%.

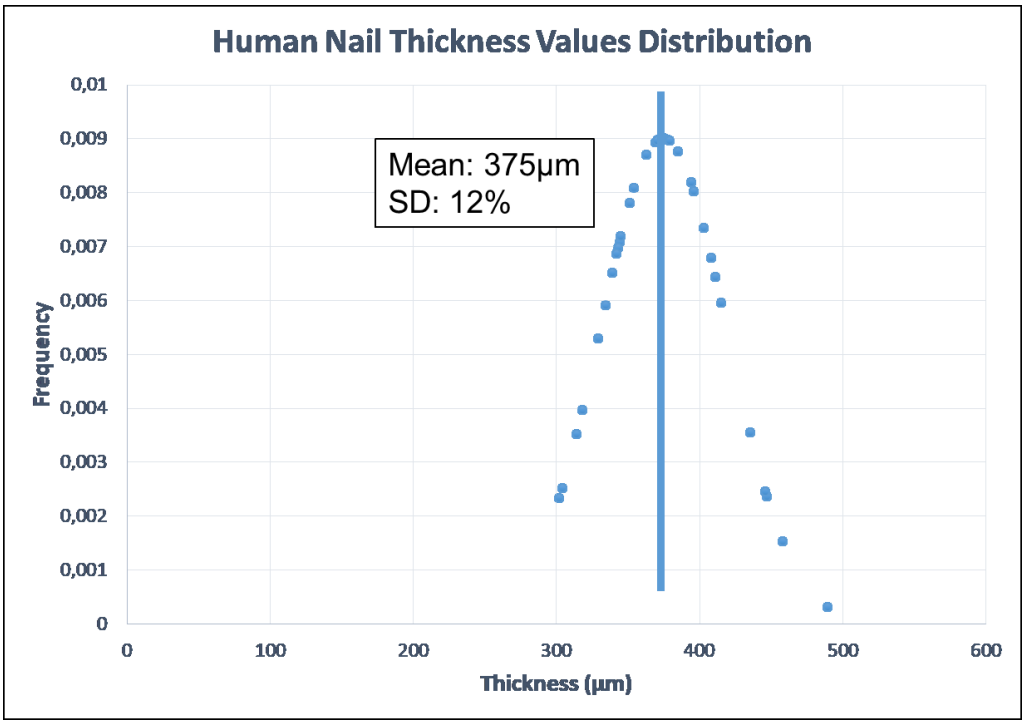

Figure 18. Thickness values distribution of human nail

In Figure 18 are reported data obtained in vivo using the same settings as for the in vitro measurements. The data reported in the graph are a total of 37 values obtained from 9 different subjects only (positions 2;3;4;7;8;9 only).
Results obtained permitted to conclude that the method described allowed to obtain membranes with specified values of thickness and diameter. In general, it is possible to say that thinner membranes production is a more sensitive process: the membranes obtain are more delicate and easily breakable in comparison to thicker samples. Moreover, a protocol to precisely assess thickness for thin membranes (~200 μm) using the Nail StrainStress meter still need to be set out.

Membranes Characterization

Superficial properties

SEM and elemental composition analysis

Here below are shown the most relevant among of the pictures taken. The images obtained confirm those reported in literature [91] [92]. Lamellar organization on the surface and stratification within the cut edge section of human nails are clearly visible (Figure 19-20-21), in contrast with hooves membranes pictures (Figure 22-23-24) which did not show the same pattern. However, the images took from the free edge of the human nail clippings (Figure 24), more polished and smoothed, seem to be more comparable to those of the membranes.
There were no significant differences among hooves membranes group and human nails samples.

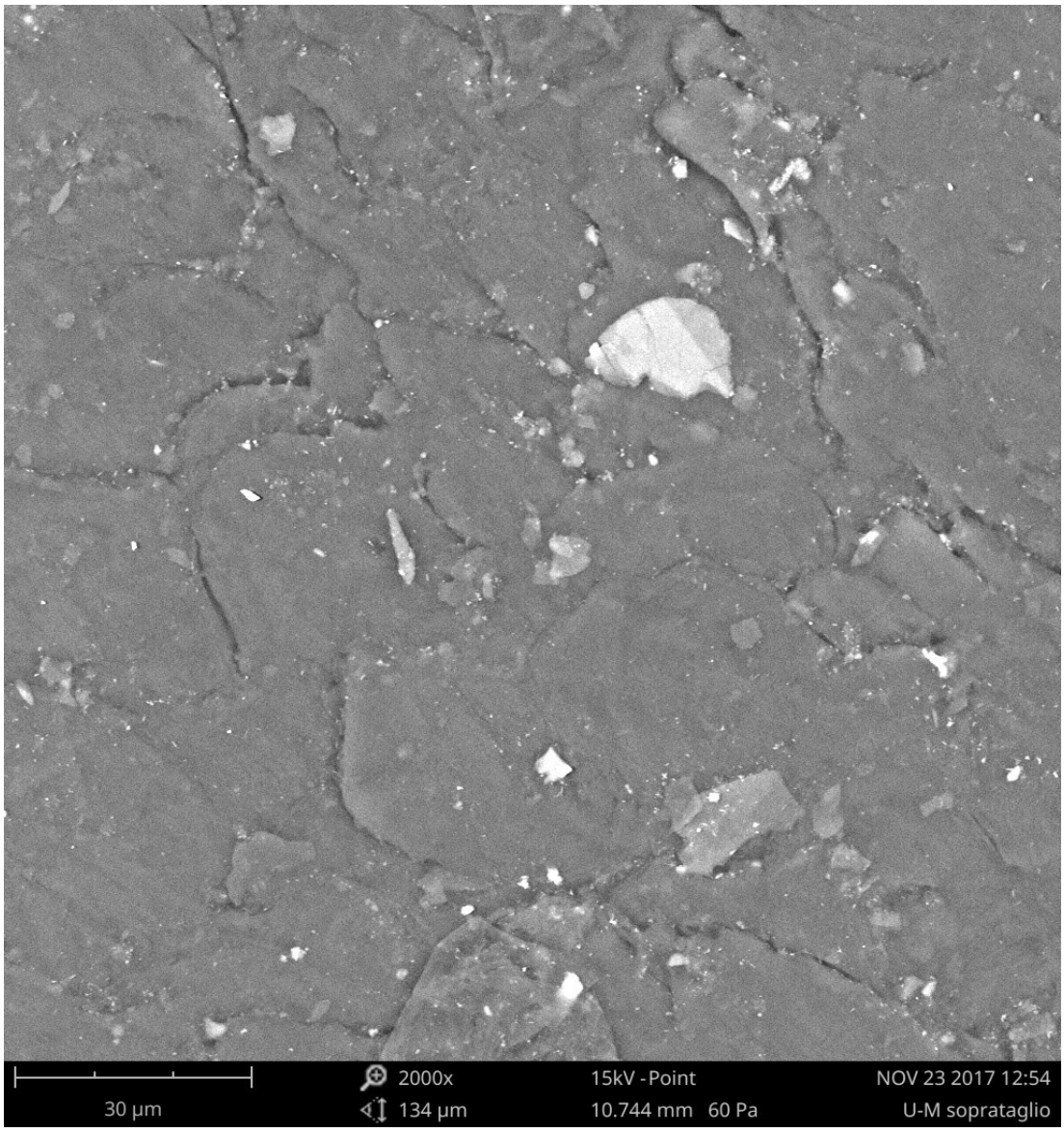

Figure 19. Human nail clipping central view (2000x).

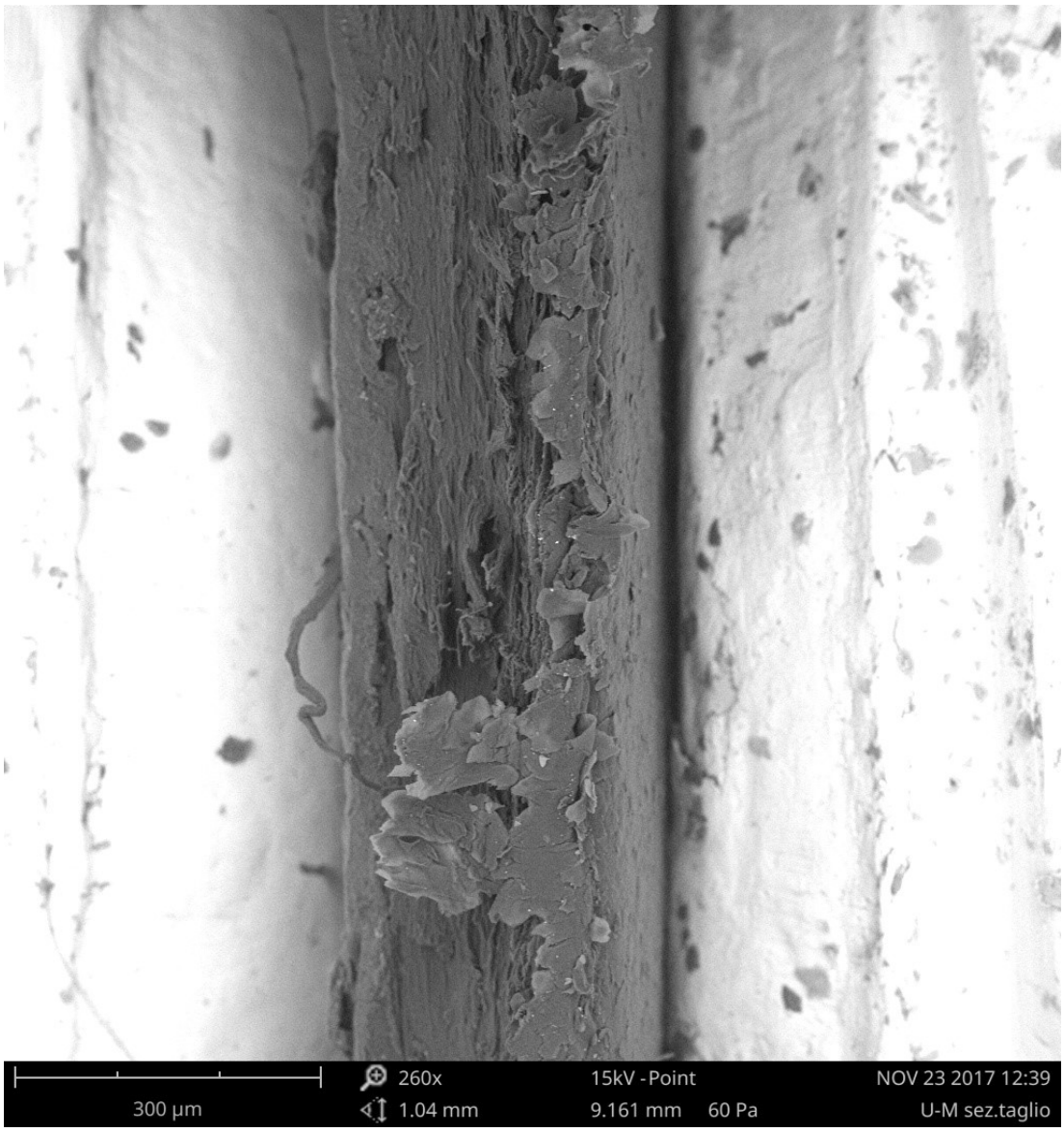

Figure 20. Human nail clipping freshly cut section view (260x).

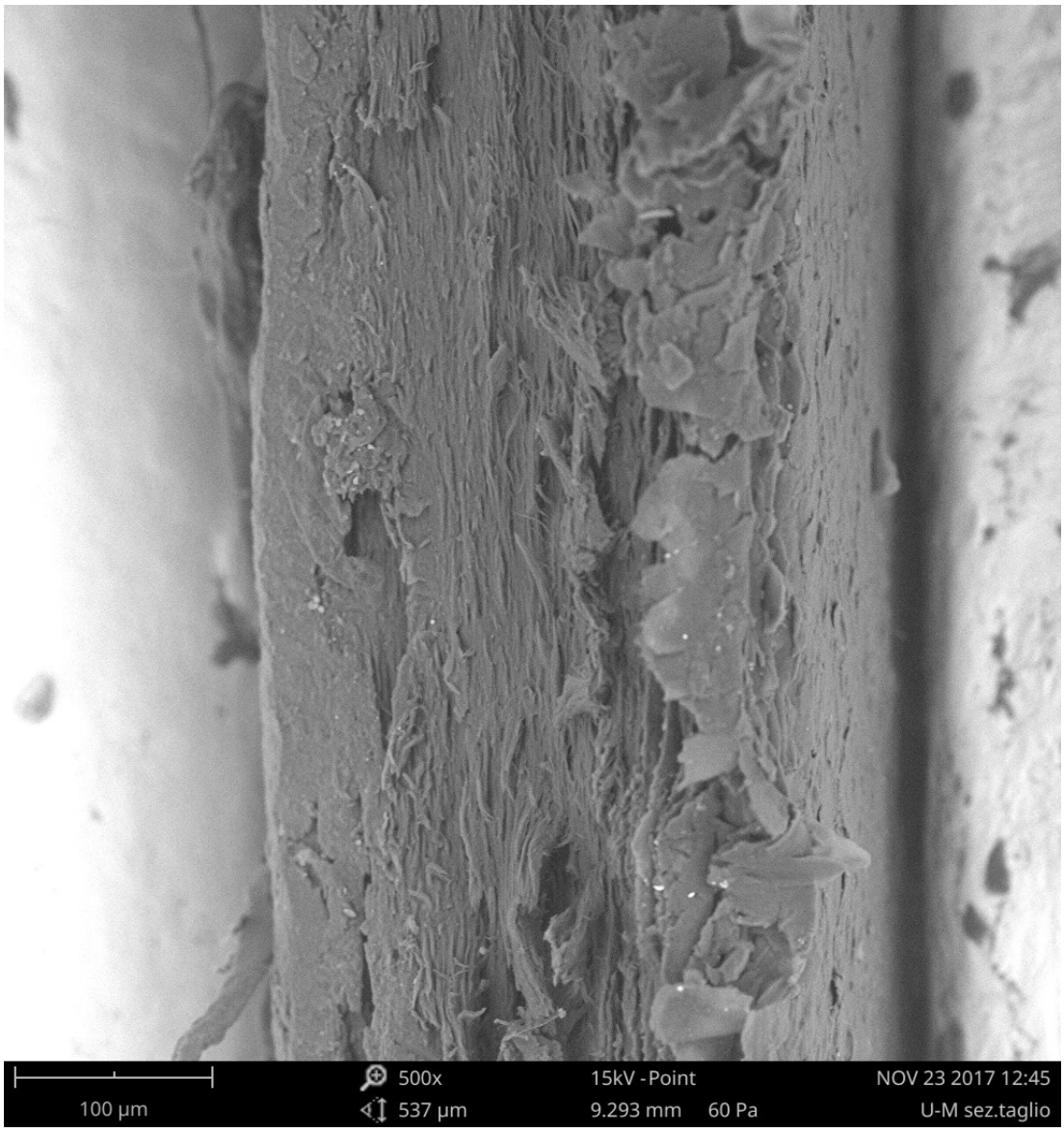

Figure 21. Human nail clipping freshly cut section view (500x).

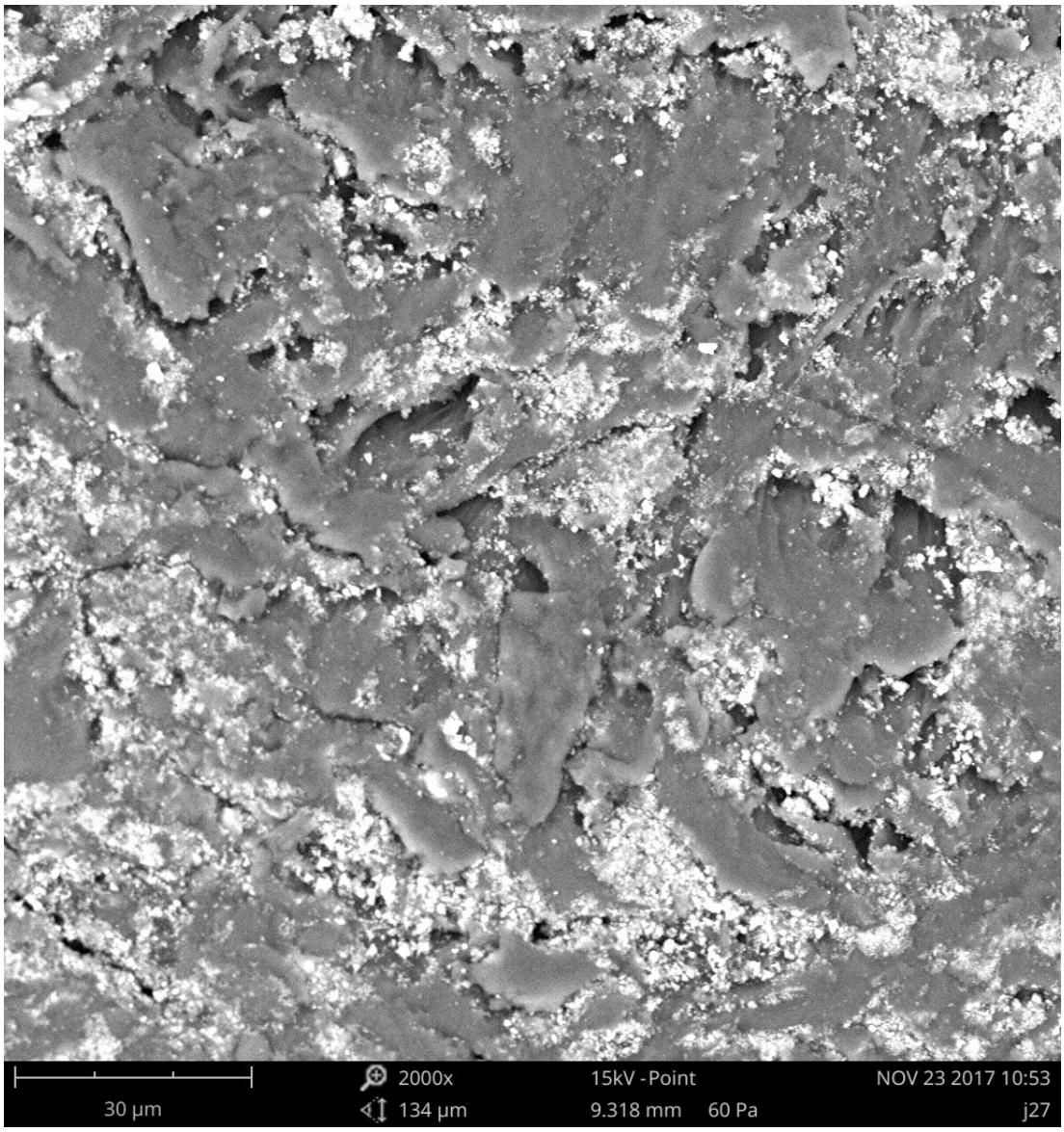

Figure 22. Hoof membrane central view (2000x).

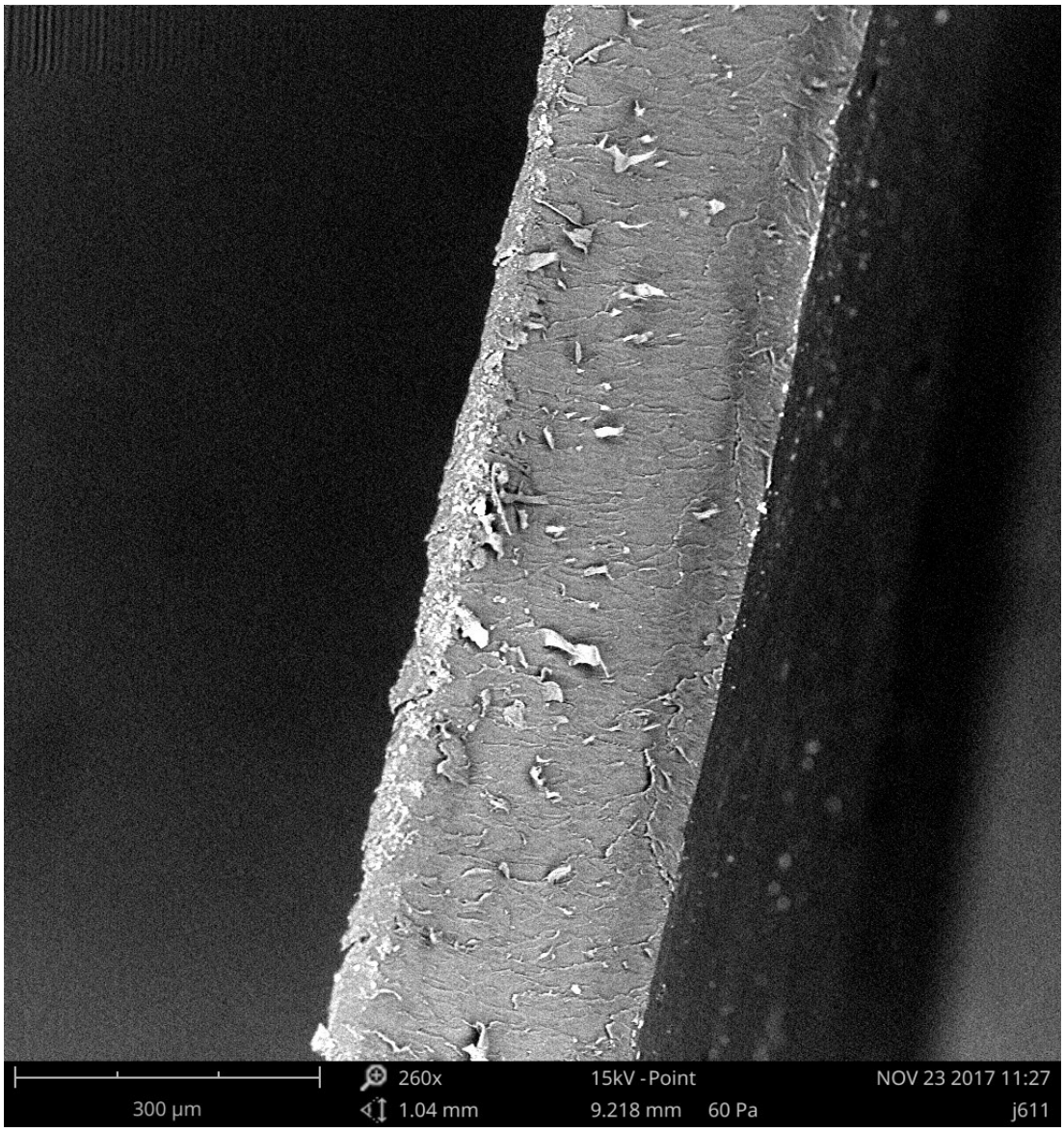

Figure 23. Hoof membrane section view (260x).

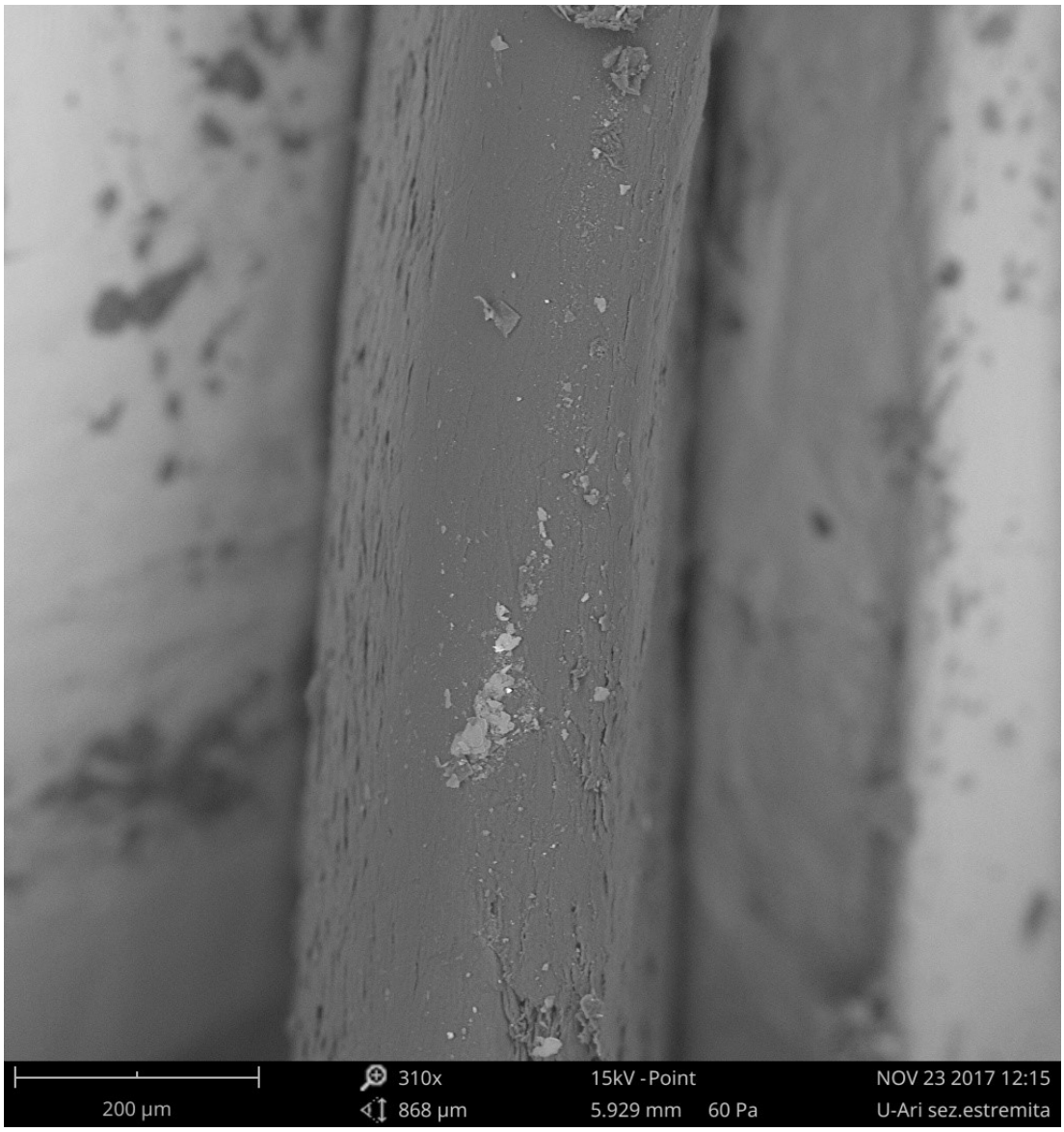

Figure 24. Human nail free edge section (310x).

The elemental analysis have been performed with a 900x magnification.
Trace elements (Br, Fe, Cr, Ni, Cl) were found only on the section of the freshly cut human nail. Since they were probably due to the cutting method, the values have been left out.
Here below a full summary of the elemental analysis (Table 7).

Table 7. Elemental composition summary

Sample	View/Location	O	C	N	S	Ca	Na	Si	Al	Mg
J.2.7	Centre	61,7	15,86	11,83	4,66	3,28	2,11	0,56		
	Edge	59,08	14,13	9,5	5,17	10,31	1,54	0,27		
J.8.1	Centre	60,66	14,98	16,59	6,65	1,11				
	Edge	60,83	16,1	13,63	7,24	2,2				
J.6.11	Section	52,52	14,01	18,15	5,57	0,88	2,04		6,84	
K.2.1	Section	53,36	25,47	8,74	3,91	3,67	2,13	2,24	0,49	
K.2.4	Centre	60,61	18,42	13,66	4,96	0,69	1,66			
	Edge	61,36	17,9	12,63	4,79	2,03	1,27			
U-M	Dorsal	57,2	16,45	11,59	5,54	0,63	1,47	0,93		0,68
	Ventral	62,44	19,01	12,77	3,62	1,68		0,48		
	Section (cut edge)	60,08	12,49	14,77	4,06	0,38	1,67		1,03	
	Section (Free Edge)	53,13	13,33	12,12	5,31	0,36	1,59	0,29	2,51	
U-A	Centre	59,67	18,44	12,51	6,6	0,98		1,07		0,74
	Cut Edge	63,24	18,29	12,07	5,02			0,74		0,63
	Free Edge	58,71	18,39	10,88	7	0,97		2,11		1,93
	Section (Cut edge)	61,45	14,84	15,79	6,58	0,23		0,38	0,74	
	Section (Free edge)	65,92	13,32	15,1	4,01	0,5		0,55	0,61	

As mentioned above, EDS allows to identify what those particular elements are and their relative proportions.

The comparison among membranes faces (Figure 25), where the means where obtained from central view and edge view values of each membrane, shown uniformity of the elemental composition, with standard deviation values below 20% with exception of the Calcium values. Definitely more variable are

the data obtained from the section (Figure 26), probably due to the different original location of the membranes (J.6.11: deep; K.2.1: superficial).

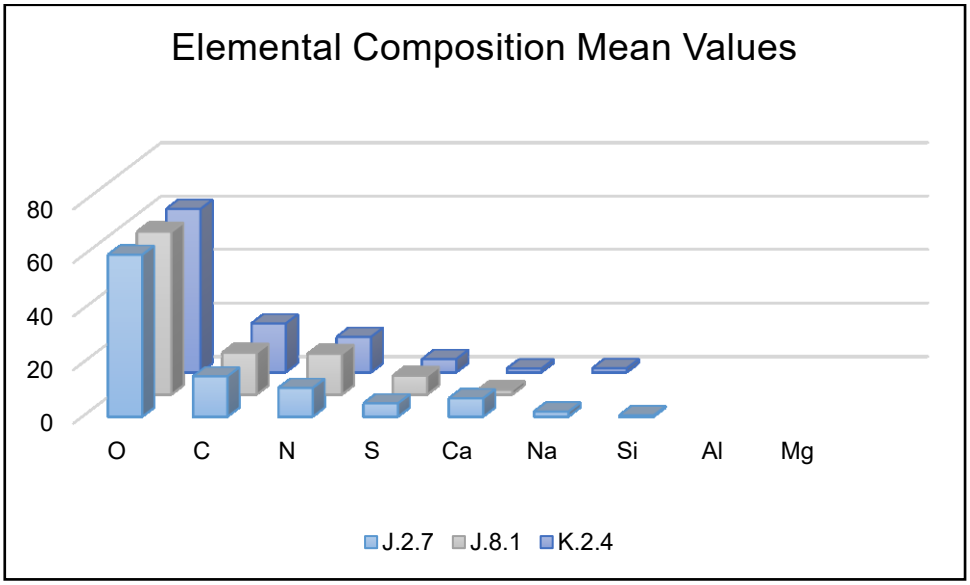

Figure 25. Membrane faces elemental composition mean values.

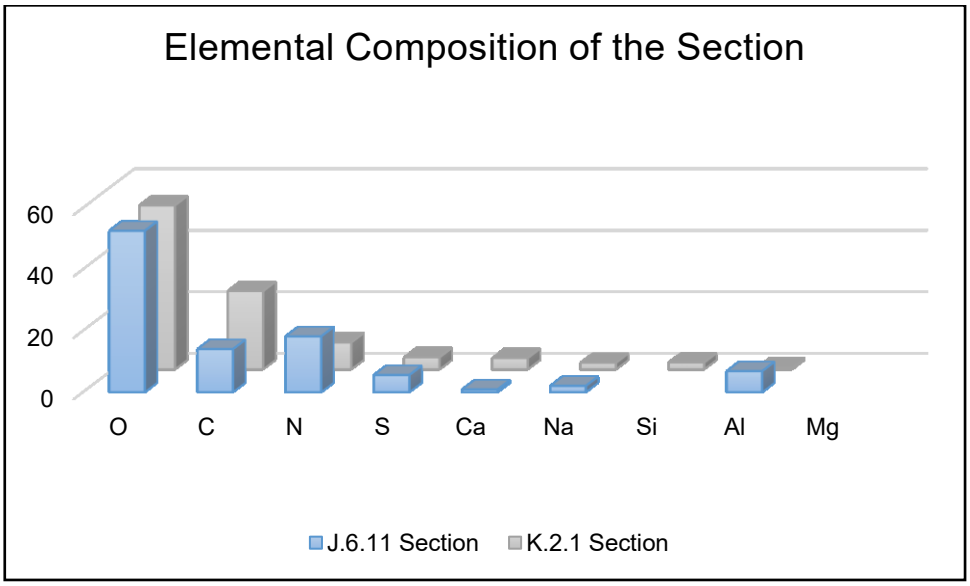

Figure 26. Membrane section elemental composition mean values.

In the graph below (Figure 27) is represented the comparison between elemental composition of both human nails and hoof membranes, and human nail and hoof membrane sections. Results highlight that the distribution of elements is quite the same between human nail and hoof membranes.

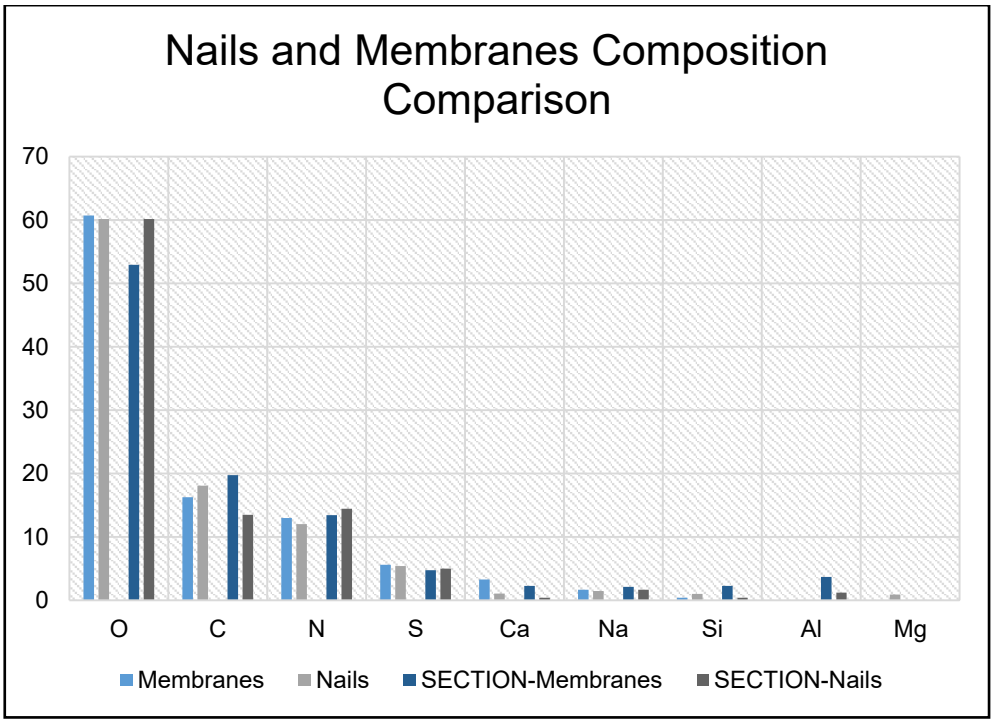

Figure 27. Comparison of membranes and nails elemental composition mean values.

Immunostaining and confocal laser scanning microscopy pilot study

After the first analysis with confocal microscope, the hoof membranes have shown a good staining; on the contrary, for human samples we had no results. No cells have been detected (HOECHST tested negative) and all the control samples tested negative.

As shown in the Figures below, no significant differences have been found between pressed and not pressed membranes nor between superficial and deep membranes.

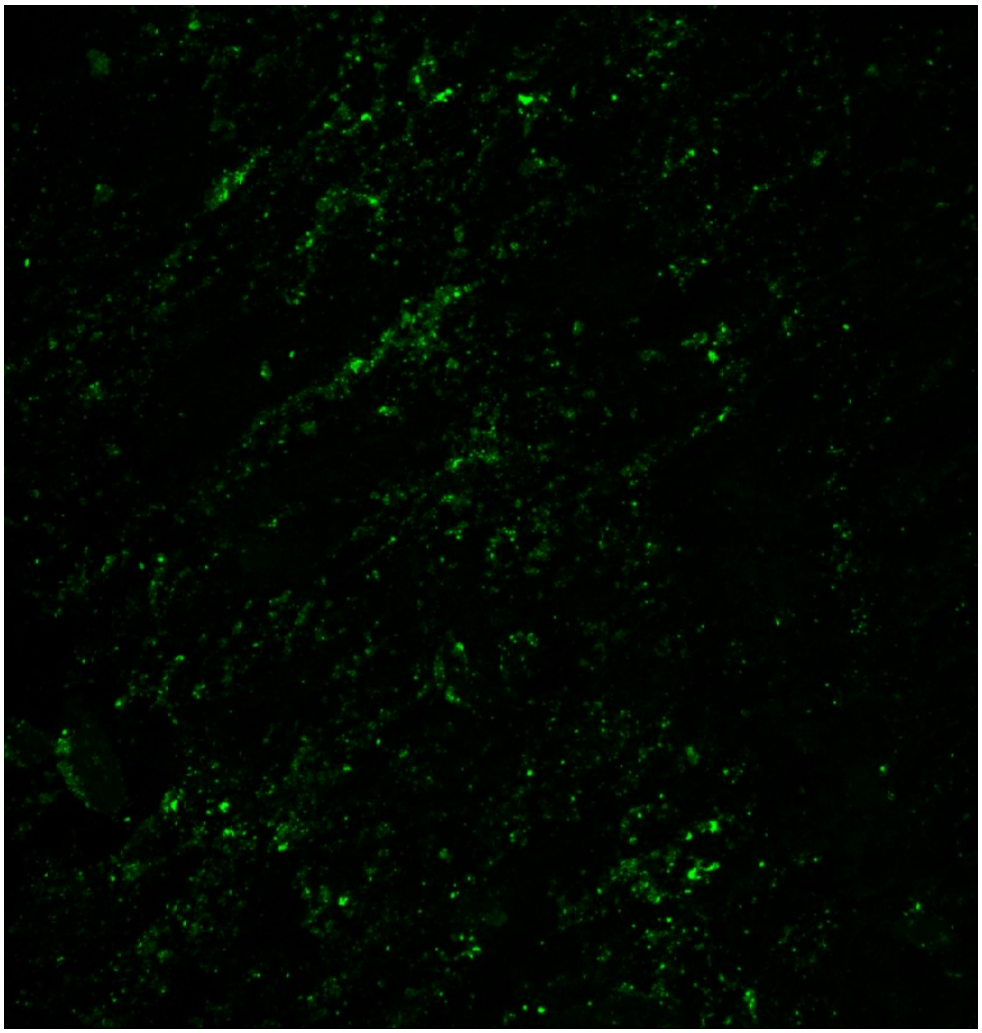

Figure 28. Sample 3.2.6 (sup/not pressed) central view.

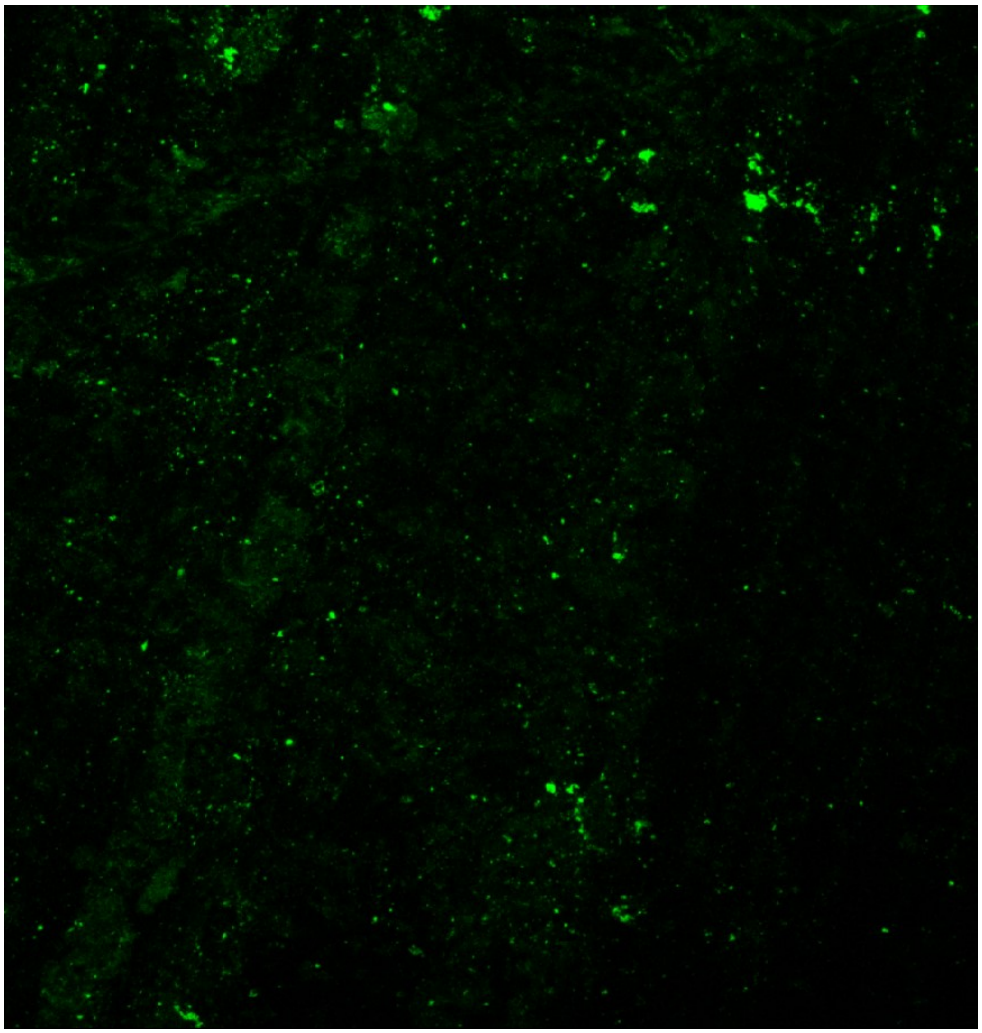

Figure 29. Sample K.2.4 (sup/pressed) central view.

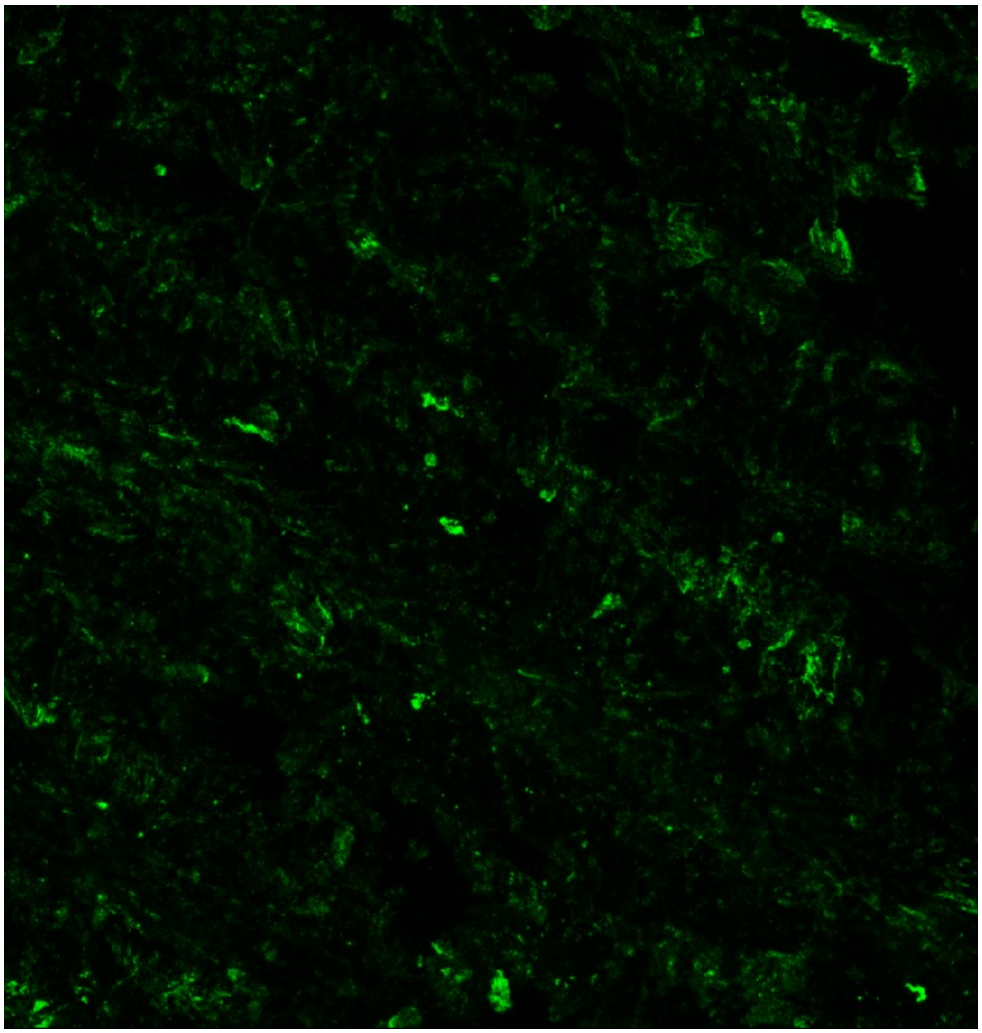

Figure 30. Sample J.6.11 (deep/pressed) central view.

Since we had no results with immunostaining on human samples, human nails have been incubated with PTA solution for 10 days to improve the permeability. Sample J.8.1 has been treated in the same way for a comparison.

As shown in the figures below the permeabilization with PTA seems to be effective. Remnants of cells (probably epithelial tissue) have been found in sample C, as shown in the Figure 31. In comparison, Sample J.8.1 (Figure 33) permeability has been far more enhanced.

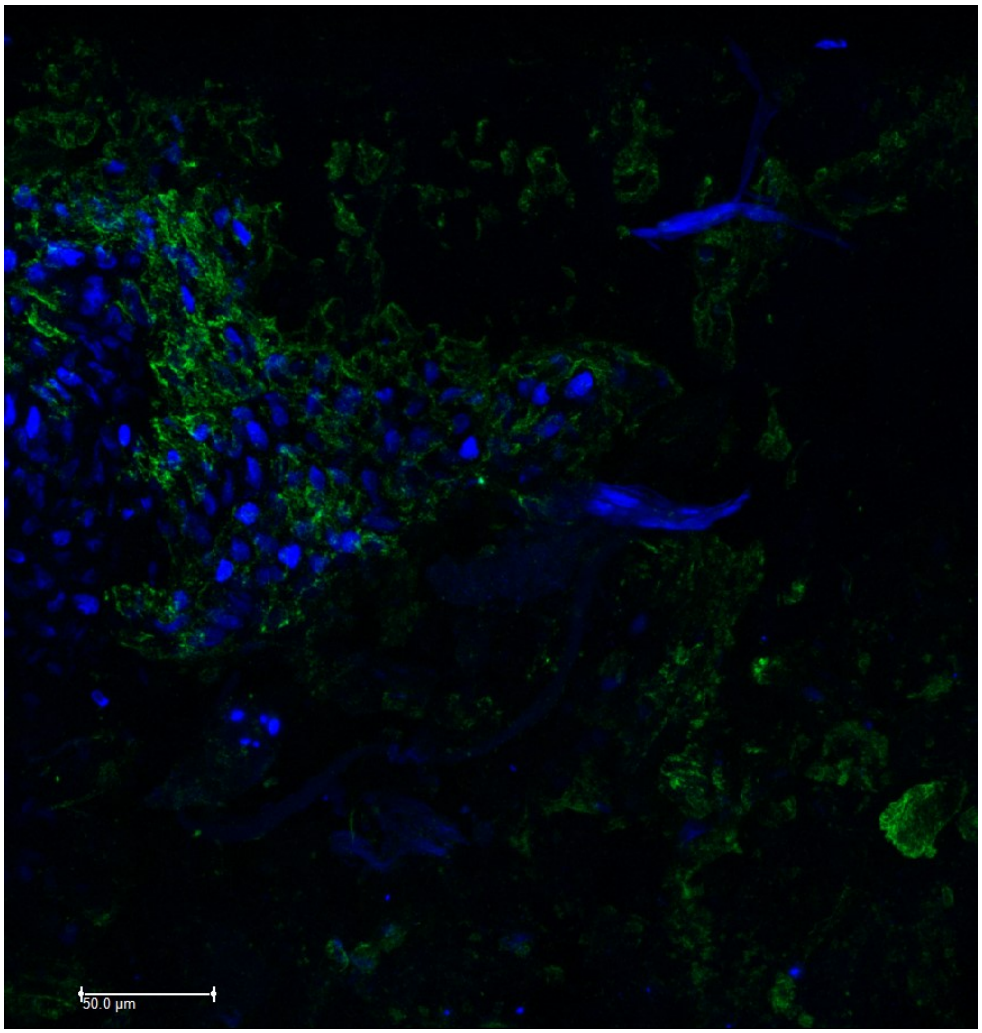

Figure 31. Sample C staining result after permeabilization procedure. Residual cells clearly visible (blue).

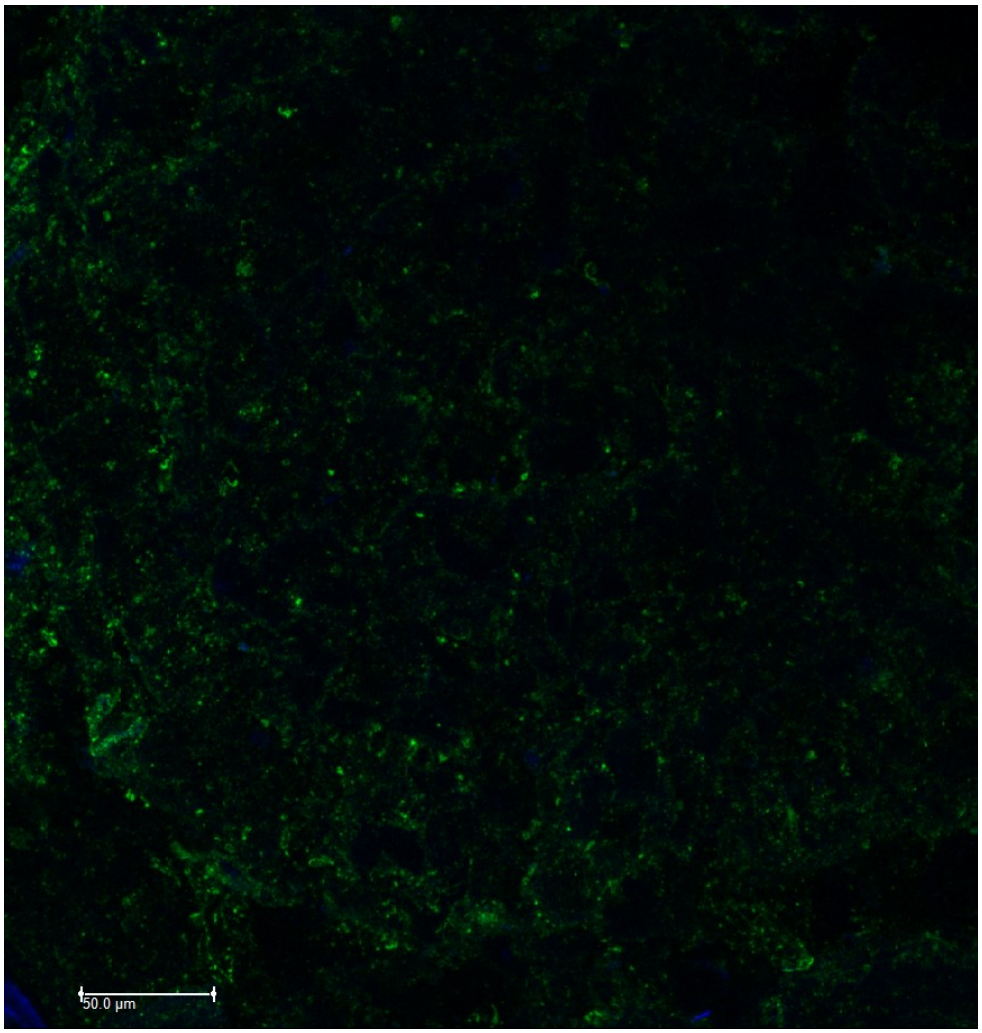

Figure 32. Sample P staining result after permeabilization procedure.

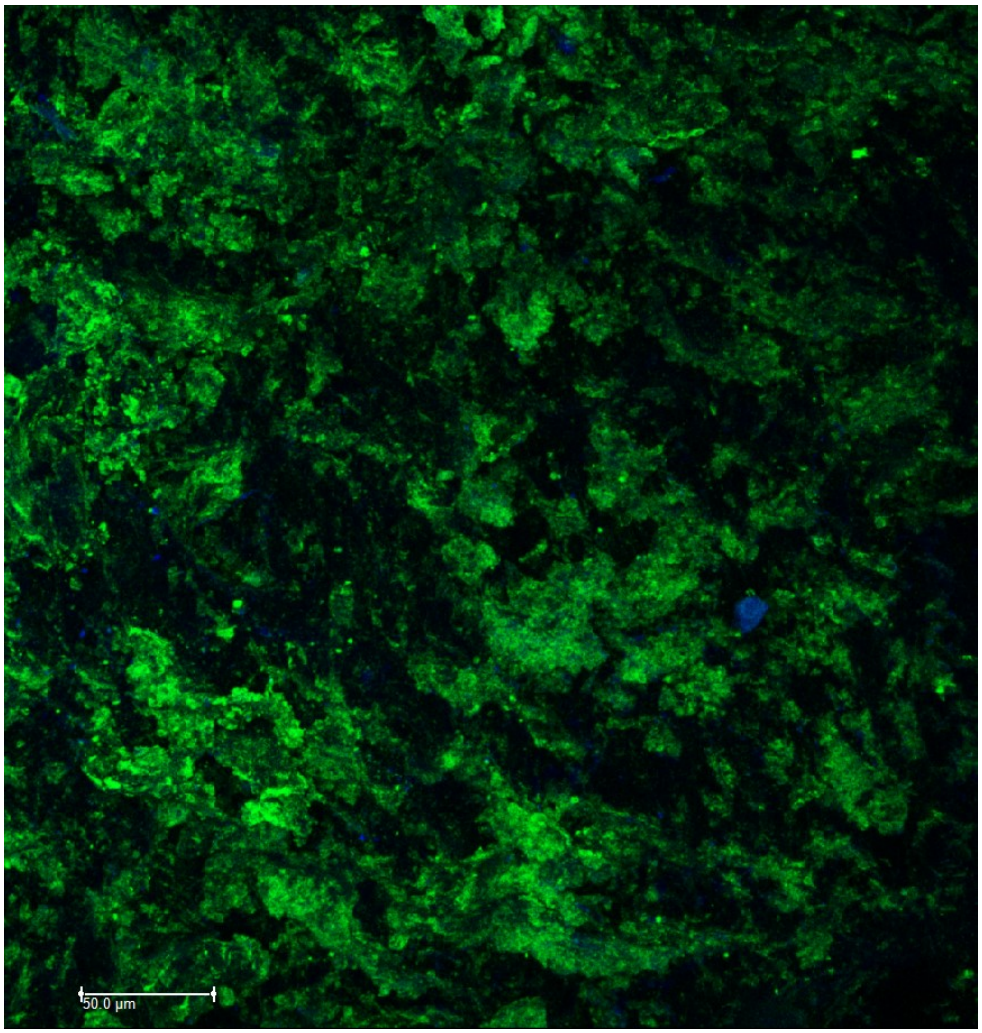

Figure 33. Sample J81 after permeabilization procedure.

Further analysis are needed to investigate similarities between human samples and hooves membranes, in particular it would be of great value a comparison between hooves membranes and pathological nail samples.

Zeta Potential and Isoelectric Point

Two human nail clippings were used for the analysis: NailG and NailA. Before loading, the samples have been washed with Ethanol 70% solution for 15 minutes and rinsed with millipore water, then cut with the scalpel to reduce the size in order to allow the loading into the cylindrical cell.

Two samples were used also to perform hooves membranes analysis, rinsed with Millipore water before loading.

The instrument settings to perform all the pH Scan analysis are reported below:

Table 8. pH scan setting.

pH Scan Settings	
Rinse Cycle	2
Zeta Cycle	2
pH Step	0.5
End pH Value	2.50

Basal condition of the human nail clippings samples at the beginning of the assay, after rinsing process, are reported in table below:

Table 9. Human nail clippings basal conditions at the beginning of the experiment, after rinsing.

Basal Condition	***NailG_1***	***NailG_2****	***NailA***
pH	9.33	9.37	9.38
Conductivity (mS/m)	18.23	19.94	20.35
T (°C)	23.62	24.07	25.29
P. Index	158	107	114
Zeta Potential	-9.23	-18.32	-7.12

***Second analysis on the same sample**

In the graph below is represented the pH dependence of zeta potential (as a function of pH in 1 mM KCl) of nail clippings samples and their Isoelectric Point (IEP).

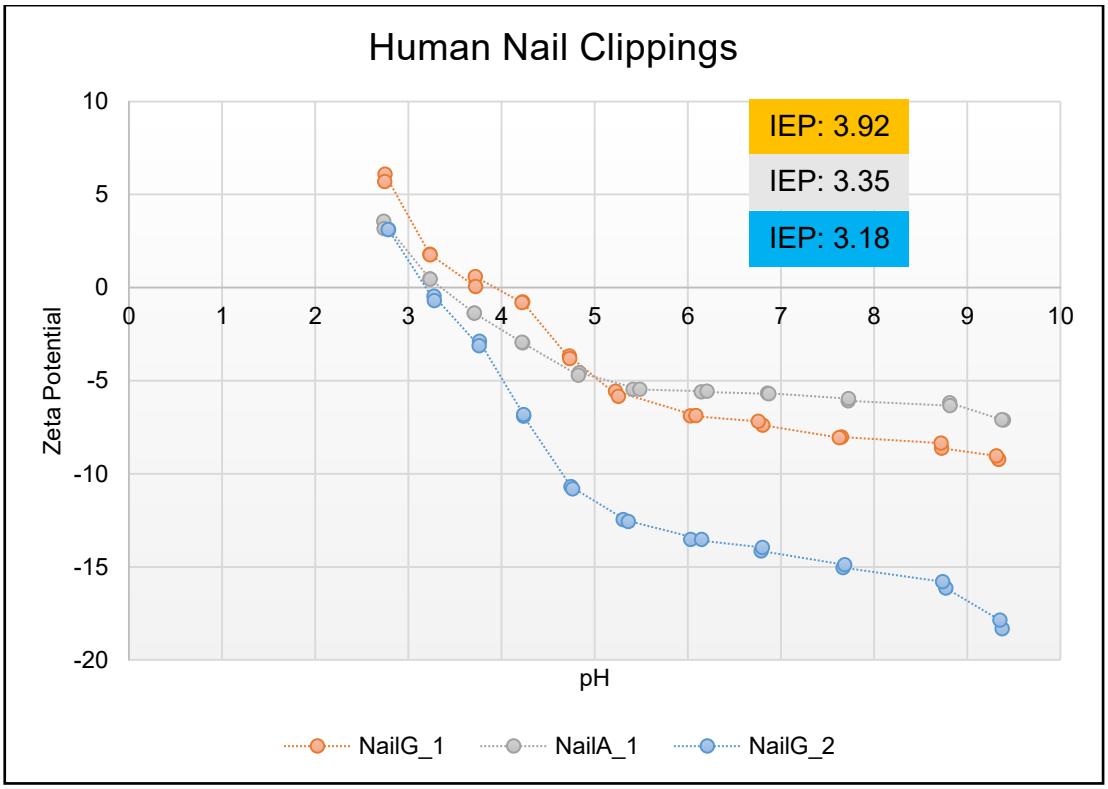

Figure 34. Human nail clippings zeta potential profiles.

Profile obtained from NailG_1 and NailA_1 pH scans are comparable while NailG_2 profile is clearly different. IEPs obtained are in a tight range even if they differ from literature reference [93].

More test with a larger sample number are required to further investigate nail surface properties. However, comparing the different profiles obtained with the two NailG analysis in this pilot study it is clear that the prolonged contact of the sample with a variable pH solution has an impact on the surface potential response.

Numerous attempts have been made to understand the best sample loading option for hooves membranes analysis. All the membranes were rinsed with Millipore water before loading.

In the graph below is reported the comparison of the profiles obtained using the standard clamping cell (20x10mm) to analyse two similar samples loaded side by side (A) and then a sample alone (B). This second option seems to be more reliable, since it allows to cover completely the sample holder surface.

Table 10. Membranes basal conditions at the beginning of the experiment, after rinsing. A=two membranes side by side; B=one membrane alone

Basal Conditions	***A***	***B***
pH	9.33	9.42
Conductivity (mS/m)	14.79	19.60
T (°C)	24.67	25.57
Gap Height	99.75	99.69
Zeta Potential	-35.32	-46.74

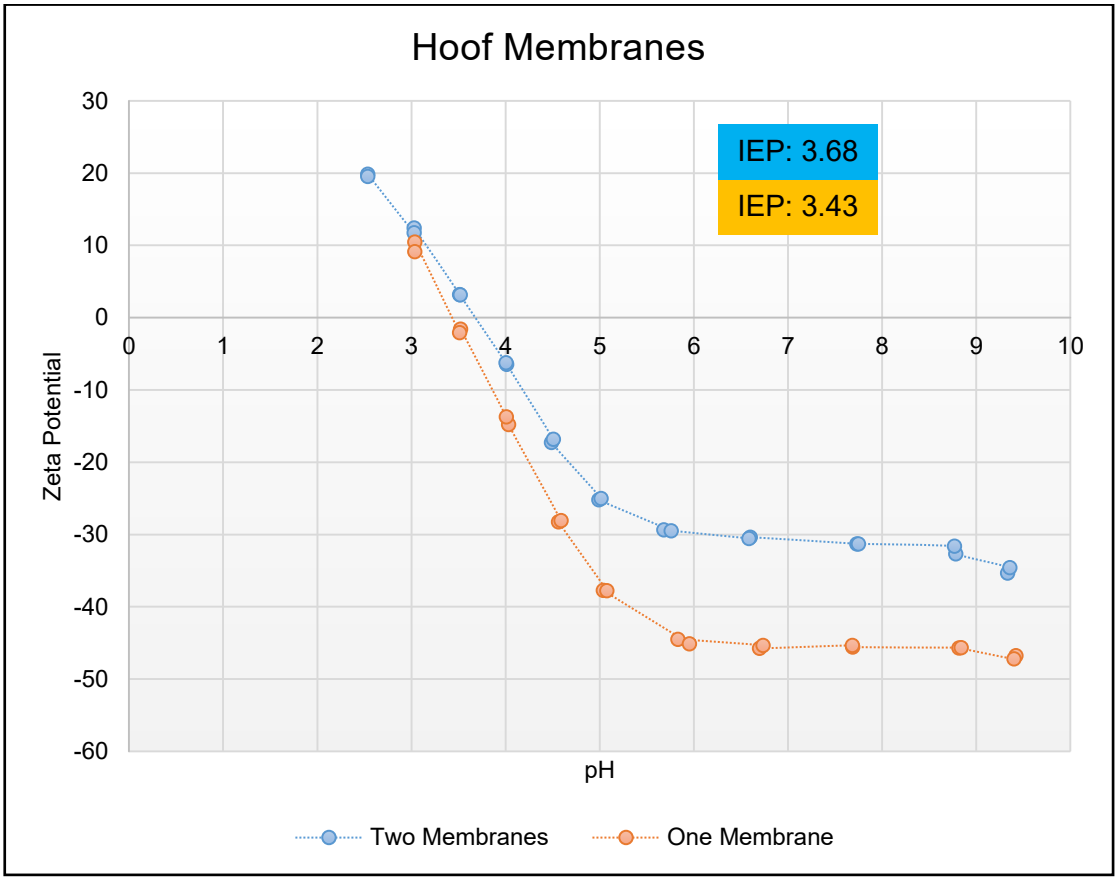

Figure 35. Hoof membranes zeta potential profile: comparison between the use of one membrane alone and two membranes in the standard clamping cell.

The profiles obtained seem to suffer the influence of the polymer of which the cell is made of, reaching strongly negative zeta potential values when expose to basic solutions.

The last assay has been performed loading a membrane on its two sides (front and back) into a customized clamping cell (10x10mm) performing a reverse scan (from pH 3 to pH 9).

Table 11. pH scan setting.

pH Scan Settings	
Rinse Cycle	2
Zeta Cycle	2
pH Step	0.5
End pH Value	9.00

Table 12. Basal conditions of the sample before the experiment after the rinsing procedure.

Basal Conditions	***Front***	***Back***
pH	3.16	3.14
Conductivity (mS/m)	47.28	50.15
T (°C)	29.52	29.31
Gap Height	102	97
Zeta Potential	4.23	3,56

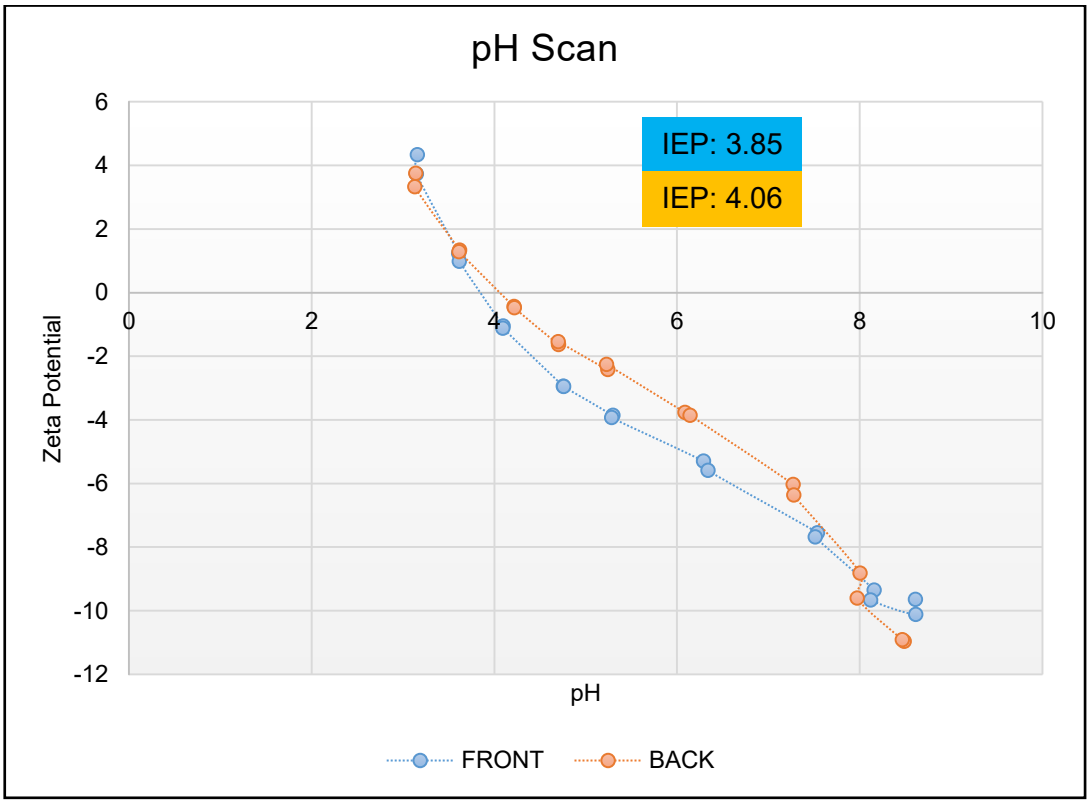

Figure 36. Hoof membrane front and back zeta potential profile using a 10x10mm customized clamping cell.

The profiles obtained with this method look more closely comparable to those of the human nails clipping.

Hoof membranes have shown to be suitable for surface zeta potential analysis: the application of this technique has a huge potential in studying effects of ingredients and products on nail, in addition or even in replacement of permeability studies, since Surpass3® is used for surface absorption studies as well. Of course, a study with larger sample numbers is needed for further investigation.

Contact Angle

Different studies, while not recently, has been performed on many aspects of this field, such as the study of the relationship between roughness of the surface and hydrophobic behaviour but there is no general consensus among this relationship [94]. In this work, the contribution of membranes thickness and origin on wettability properties has been analysed.

A total of 71 samples have been analysed to evaluate wettability properties of flaky hoof membranes with water. Of these membranes, 49 were thin membranes (~200 µm) and 22 were thick membranes (~300 µm). In table 13 are reported the total mean of the CA obtained at T0 along with the percentage standard deviation (%SD).

Table 13. Summary of mean T0 CA results (N=number of sample)

	200 µm	300 µm	Total
N	49	22	71
Mean	65,54	79,13	69,75
%SD	22,20%	21,12%	23,52%

Considering all the samples, flaky membranes population are divided with 28 samples with a CA < 65° (hydrophilic) and 43 showing a CA > 65° (hydrophobic). Analysing the data by thickness, T-test result has shown that between thick membranes (mean T0 CA=79,13°) and thin membranes (mean T0 CA=65,54°) there is a statistically significant difference (**p=0,002).

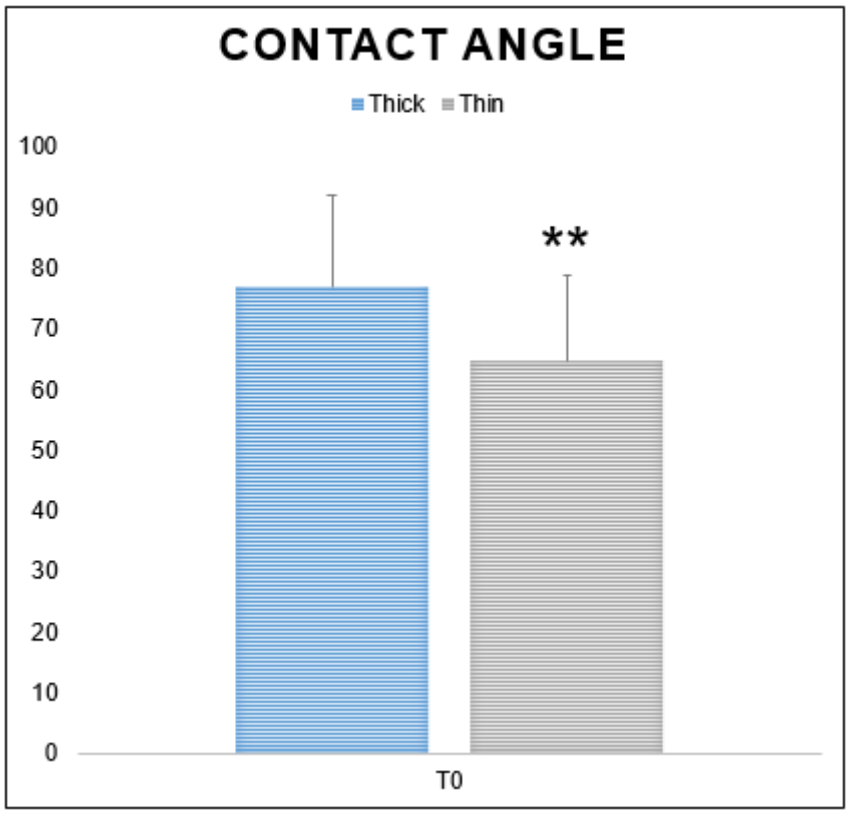

Figure 37. Contact angle mean value: comparison between thick and thin membranes.

However, looking at the reduction of the contact angle in time, there is no difference (p=0,54) between thin (59,11% reduction of CA value over time) and thick (54% reduction over time) membranes. In summary, thickness seems to have impact on the absolute contact angle value itself, with thin membranes more wettable/hydrophilic than thick membranes.

The thin membranes subset has been analysed separately to evaluate a possible correlation between the contact angle absolute value and the origin of the membrane in terms of depth. The values are relatively homogeneous among central membranes, while the most superficial and deep ones distance themselves more, showing lower CA values, and so a higher wettability of the surface. The trend of the CA in relation to the origin of the membranes is showed in Fig. X. although a larger sample group is needed to have a more robust conclusion.

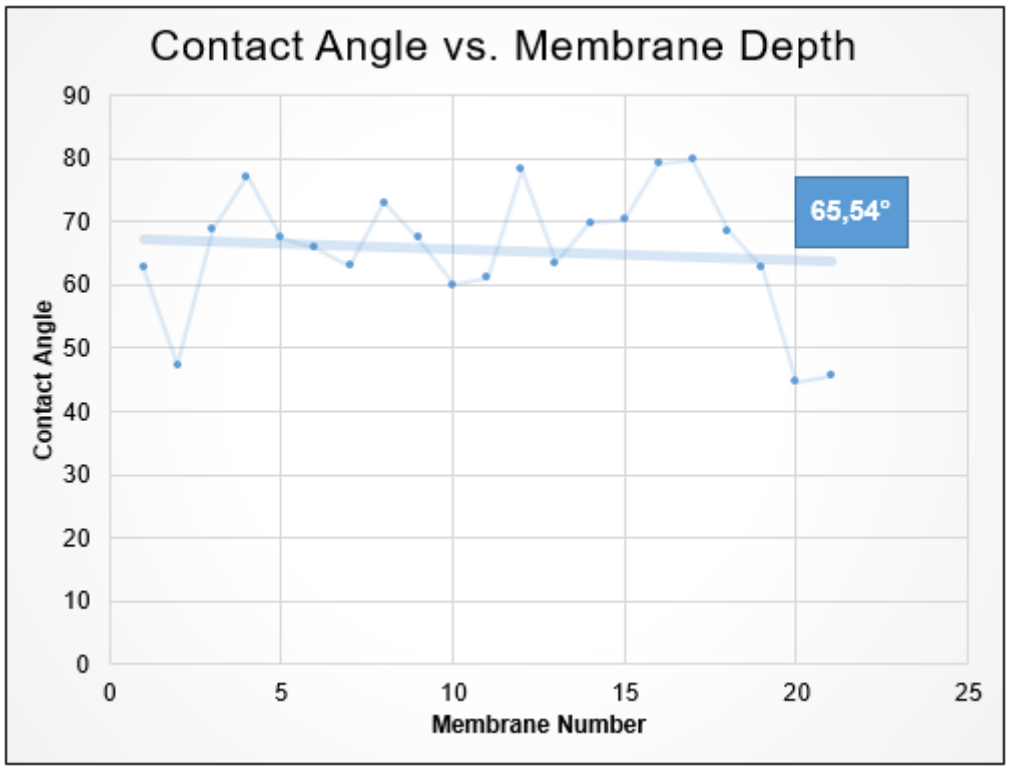

Figure 38. Thin membranes subset: CA values in relation with membranes depth. Mean=65,54°

The application of this assay could have a dual utility. It could be apply as a characterization method to select membranes which have a compatibility with the solvent used in the assay: for instance, before a permeability study it can be use to assure that the vehicle used would not be a rate-limiting step in the active ingredient permeation. In addition, it could be a method to assess the effects of ingredients and products on the nail, giving information on how the surface would or would not be modify by the tested substance, comparing the surface wettability properties before and after use.

Mechanical Properties

More than 300 membranes in the thickness range of ~300µm were mounted on custom made PTFE support and analysed with Nail StrainStress Meter NM100 to evaluate the suitability of the hoof membranes for mechanical properties evaluation and to study the relation among parameters obtained. As mentioned before, all membranes have been kept in the climatic chamber at controlled conditions (T=25°C, RH=40%), and mechanical properties

measurements were made in an air conditioned room (T 25°C, R.H. 50 ± 5%). Curvature and nail length in human subjects are highly variable among volunteers. Moreover, human nails are constantly growing, so the evaluation of mechanical properties in a in vivo long term study on safety and efficacy of ingredients or products could be difficult. Working with in vitro model makes it possible to overcome these potential issues from the beginning. The hoof membranes were mounted on the supports with a constant portion of sample exposed (5mm). In this way, the sample length will not have an impact on mechanical parameters evaluation and results.

Reproducibility Test and Water Effect

20 membranes classified as flaky or flaky/smooth have been selected for the reproducibility test. In Table 14 are reported the mean values of the measured mechanical parameters (VI, FI and Thickness) at the three time points of analysis: T0, T1 (after 24hrs) and T2 (after 1 week) and the relative percentage standard deviation (%SD).

Table 14. Mean values obtained for each mechanical parameter and their relative percentage standard deviation (%SD).

	VI		FI		Thickness	
Sample	**Mean**	**%SD**	**Mean**	**%SD**	**Mean**	**%SD**
1.15.9	135,85	8,23	199,09	0,65	327	1,24
3.11.1	147,58	6,24	110,57	1,29	322	4,70
1.15.10	189,37	2,33	93,38	10,28	294	3,93
2.6.11	222,80	4,86	135,82	2,49	285	6,33
A.3.2	77,18	3,41	149,45	4,48	325	3,39
2.4.13	191,57	5,97	75,03	21,20	274	1,93
1.6.5	83,70	8,87	173,72	8,52	295	5,25
1.15.11	156,12	11,68	106,25	1,36	311	5,75
1.6.6	128,05	11,97	243,91	10,44	328	6,14
2.2.4	65,79	1,84	168,82	13,35	378	8,23
A.10.8	182,90	3,60	152,90	11,60	309	4,08
A.8.13	157,44	4,03	76,28	11,38	282	7,03
4.13.10	196,34	7,80	148,11	13,80	273	4,50
4.13.6	63,23	3,16	87,14	10,34	314	4,93
2.2.6	73,07	0,48	179,44	4,00	311	3,75
4.18.5	262,31	7,31	42,45	14,30	275	7,82
A.10.10	78,71	3,72	179,44	13,42	291	3,31
1.15.3	96,32	6,79	76,15	14,73	374	5,11
1.9.9	175,34	15,98	199,63	10,91	291	3,90
1.2.6	60,21	4,34	124,19	10,79	358	2,94
MEAN		**6,13**		**9,47**		**4,71**

The measures showed a good reproducibility. As shown in the table, the mean percentage standard deviation values for the three measured mechanical parameters are below 10% (Mean %SD: VI = 6,13%; FI = 9,47%; Thickness = 4,71%).

The water effect on mechanical properties of membranes has been evaluated measuring all the parameters before and after soaking 19 membranes in warm water for 10 minutes (37°C). As for the reproducibility test, the membranes selected for the experiment were all classified for morphological properties as flaky or flaky/smooth. In the table below are reported the results expressed as the mean % variation among the parameters at the different time points of analysis: T0=beginning; T1=right after soaking in water; T2=24 hours of drying time; T3=72 hours of drying time.

Table 15. Mean % variation of mechanical parameters at different time points after soaking the membranes in warm water

%Variation	VI	FI	Thickness
T1 vs. T0	237,39	-45,59	9,84
T2 vs. T1	-66,12	79,81	-5,27
T2 vs. T0	2,44	-8,25	3,76
T3 vs. T2	-1,83	33,74	-0,91
T3 vs. T0	-1,38	0,08	2,49

Soaked membranes (T1) showed a major variation of all mechanical parameters: on average, the Viscoelasticity Index (VI) increases its value of about +237,39%, while the Firmness Index (FI) decreases of about -45,59%. Thickness is coherently increased with a mean of 9,84%, due to the water retained by the membranes. The drying time process has been shown to be very heterogeneous among the 19 membranes. However, after 72 hours of drying time in controlled conditions (T3), the parameters returned to values very close to the initial ones (mean % variation T3 vs. T0: VI=-1,38%; FI=0,08%; thickness=2,49%). To confirm the reproducibility of the analysis, all the mechanical parameters have been measured again after 1 week of drying time (T4) in controlled conditions. All the mean values for each sample obtained for the three parameters at T0, T3 and T4 are reported in the table below, along with the % standard deviation (%SD).

Table 16. Mean values obtained for each mechanical parameter and their relative percentage standard deviation (%SD).

	VI		FI		Thickness	
Sample	**Mean**	**%SD**	**Mean**	**%SD**	**Mean**	**%SD**
1.10.4	127,33	4,40	125,52	3,84	309	3,90
1.15.8	107,43	5,29	204,50	1,53	302	1,26
1.6.12	157,46	6,59	113,15	7,36	315	2,52
1.6.3	104,48	2,57	214,56	4,36	303	4,58
1.6.4	98,20	0,53	178,65	1,50	327	0,18
1.6.8	191,69	4,63	145,22	3,62	262	5,97
2.10.4	55,09	0,63	129,94	3,92	333	2,57
2.3.6	227,49	6,60	105,12	6,22	281	3,04
2.6.12	71,22	10,12	84,91	4,80	379	2,94
2.6.5	156,80	1,53	215,02	4,37	255	4,31
2.7.15	144,38	2,46	117,84	5,60	306	2,90
2.9.14	132,10	0,53	214,72	11,61	275	3,17
2.9.16	87,64	2,46	145,87	5,51	347	1,16
2.9.5	93,90	0,97	99,97	0,48	321	3,15
4.10.7	69,81	1,71	167,59	4,56	323	7,93
4.8.6	76,29	1,85	291,75	3,11	365	1,45
A.3.5	51,33	4,47	156,56	7,84	438	7,25
A.8.5	116,74	0,84	271,62	4,33	304	2,76
A.8.9	114,21	1,49	122,23	1,11	321	1,36
MEAN		**3,14**		**4,51**		**3,28**

As the results shown, the mean %SD is lower than 10% for all the parameters evaluated (VI=3,14%; FI=4,51%; Thickness=3,28%). This data confirm both the reproducibility of the measures and the complete recovery of the membranes after the soaking process. 3 days have been chosen as the standard drying time and conditioning time for membranes before being analysed.

Correlation Analysis

In table 17 are reported the mean values of the three analysed parameters obtained from a total of 265 hoof membranes and human volunteers (9 subjects; 37 values; position 1; 5; 6; 10 were excluded): Viscoelasticity (VI), Firmness Index (FI) and Thickness.

Table 17. Mechanical parameters mean values and percentage standard deviation values (%SD) for hoof membranes and human nails.

	Hoof Membranes		Human Nails	
	Mean	**%SD**	**Mean**	**%SD**
VI	110,111	36,630	71,034	27,803
FI	118,896	43,748	171,105	43,665
Thickness	320,3	15,7	375,8	11,8

In comparison to what has been shown for the thickness values, the VI e FI data have a wider distribution around the mean value, both for hoof membranes and human nails. Either parameters showed a normal-like distribution.

The difference between the main value of mechanical parameters is no surprise, since hoof membranes are generally described as more flexible and porous than human nails. Moreover, the assay in vivo could be conditioned by the surface of the sample expose, meaning the nail length. For hoof membranes the length is standardize on 5mm while for human nails of the volunteers varies from a minimum L1=1,11mm to a maximum of L1=4,49mm. The influence of the length is still unclear, however to eliminate the bias before an experiment it could be necessary to start from people with nails the same length range and to perform the mechanical assay at the different time point taking onto account the nail growth. Of course, with in vitro model it is possible to standardize the condition easily, working on the same surface of the sample for the all duration of the experiment.

Another critical factor to be considered is that the human subjects who volunteer for the assay were all 14-30 years old, they were all healthy with no detected disorder or pathology. Thus, the values obtained through this in vivo mechanical assay reflect a gold standard of healthy nails, while the hoof membranes in vitro model should reflect the nail characteristics within the broad spectrum of disease affecting nail mechanical properties, from brittle nail syndrome, to onychomycoses. A higher number of healthy subjects as well as

an in vivo assay on subjects affected by nail pathologies would be necessary to make these assumptions more solid.

The next step has been the comparison of the mechanical parameters obtained on hoof membrane to understand the existing relationship among them. The pictures below report the scatter plots of the different mechanical parameters. No data have been left out in this analysis.

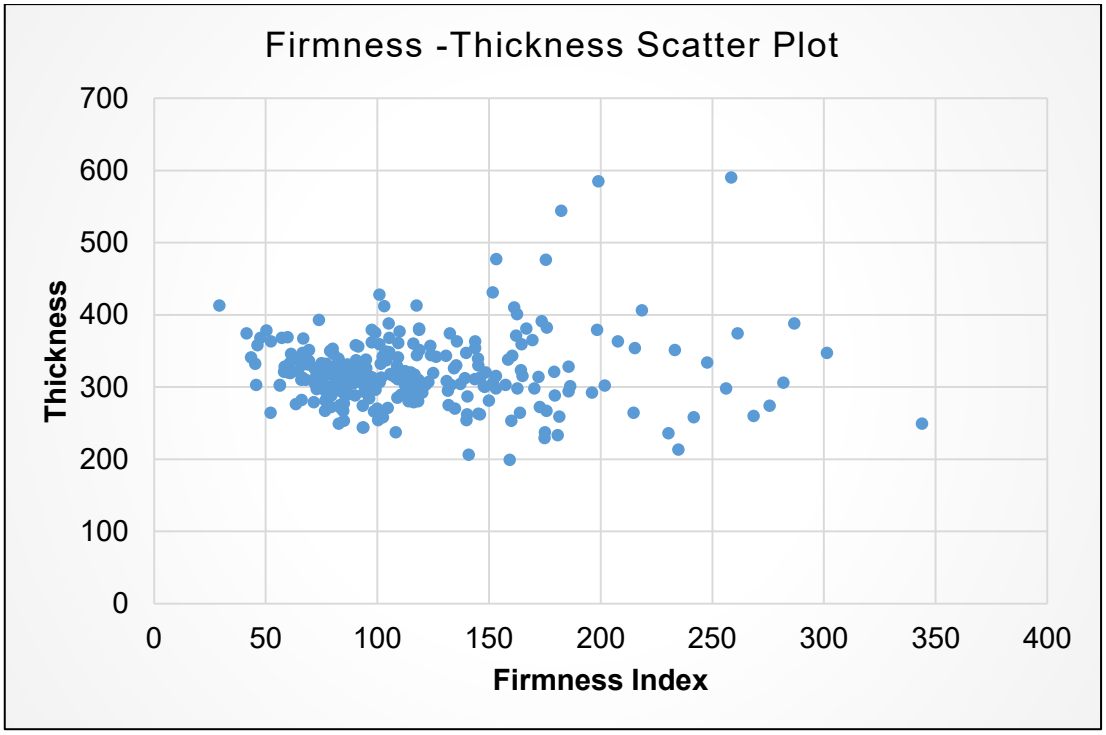

Figure 39. Correlation between Firmness Index and Thickness values

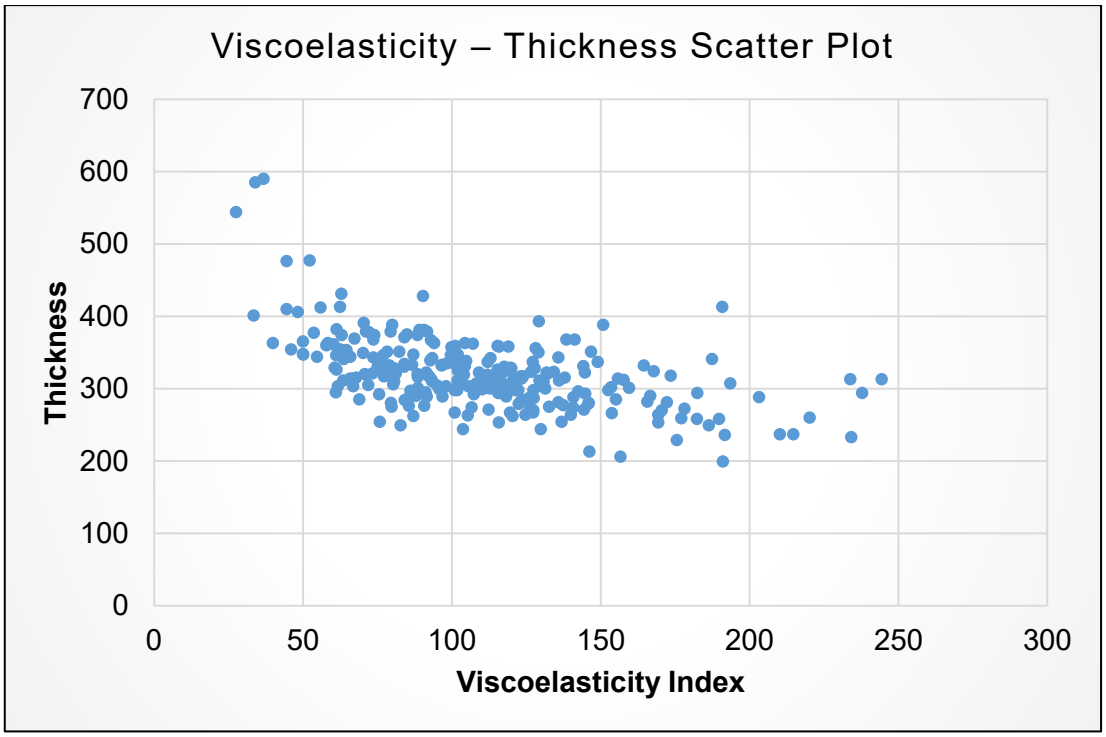

Figure 40. Correlation between Viscoelasticity Index and Thickness values

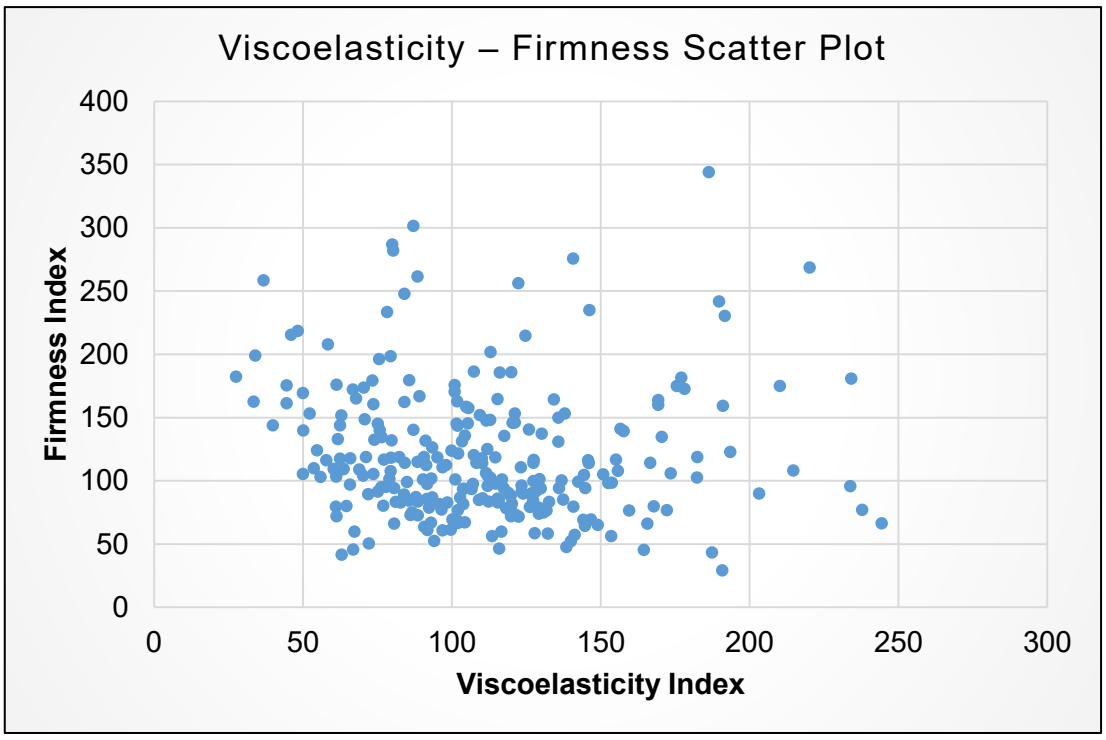

Figure 41. Correlation between Viscoelasticity Index and Firmness Index values

Pearson's and Spearman's rank correlation coefficients have been calculated for all data set as reported in the table below.

Table 18. Correlation coefficients comparison

Correlation Coefficient			
	Pearson's	Spearman's Rank	Strength Interpretation
FI vs Thickness	0,0483	-0,0485	None
VI vs Thickness	-0,536	-0,524	Moderate/fair
VI vs FI	-0,0743	-0,171	None/Poor

The graph in figure 40, representing the scatter plot of Viscoelasticity index and related Thickness values, seems to indicate a trend of inverse correlation between the two parameters, where at higher thickness values correspond a lower viscoelasticity index: thicker membranes are more rigid. Both Pearson's and Spearman's coefficient values in fact confirms this correlation respectively equal to -0,536 and -0,524. Moreover, the two values do not differ from each other, evidence that outliers and not-normal distribution of the data should not affect the result.

Firmness Index and Thickness are clearly not correlated, both looking at the scatter plot and at the calculated correlation factors reported in table 18.

The relationship between Viscoelasticity Index and Thickness Index appears to be less obvious: the two correlation coefficients are both low in term of absolute values, but different from one another. Taking into consideration the Spearman's index which should partially bypass errors due to outliers, the two parameters show a poor inverse correlation: at higher FI values correspond a lower VI, so a membrane with a high inner strength should also be more rigid. Still, the relationship is poor.

The hypothetical existing correlation between VI and FI values and the membrane depth has also been investigated. Here below are showed the relative scatter plots. In the graph is reported the mean value of the index for every membrane within the same group (1=more superficial; 17=more deep) with at least three membranes for each group (n=260).

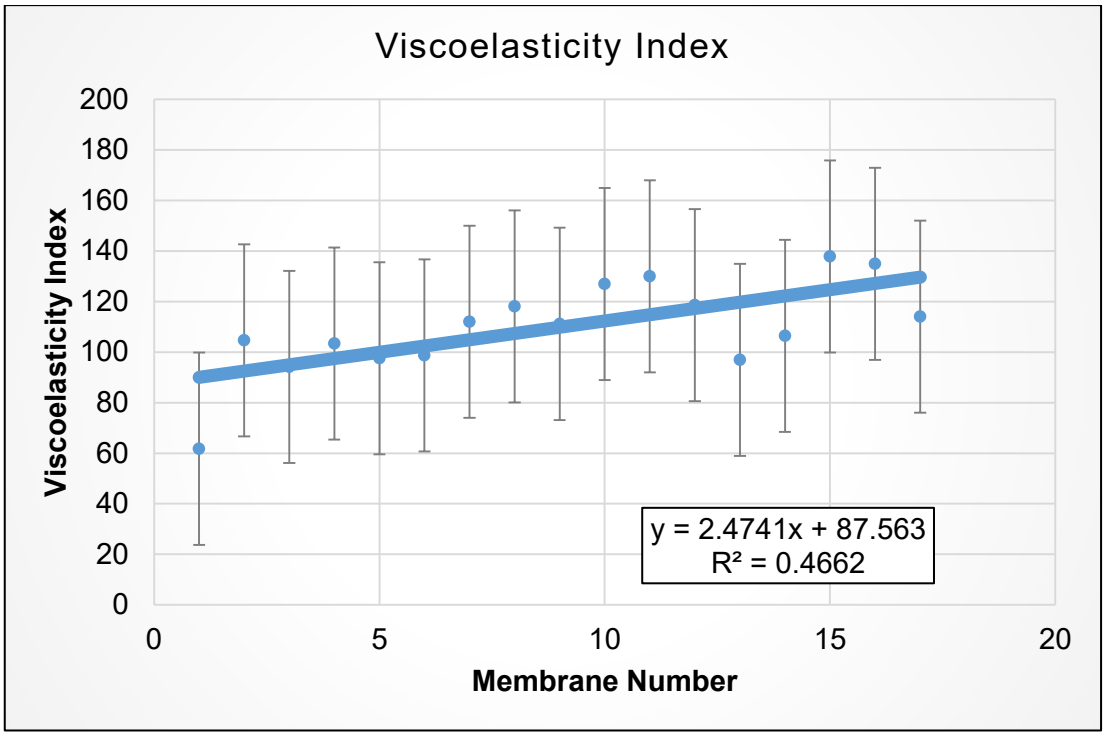

Figure 42. Correlation between mean VI values and membrane depth

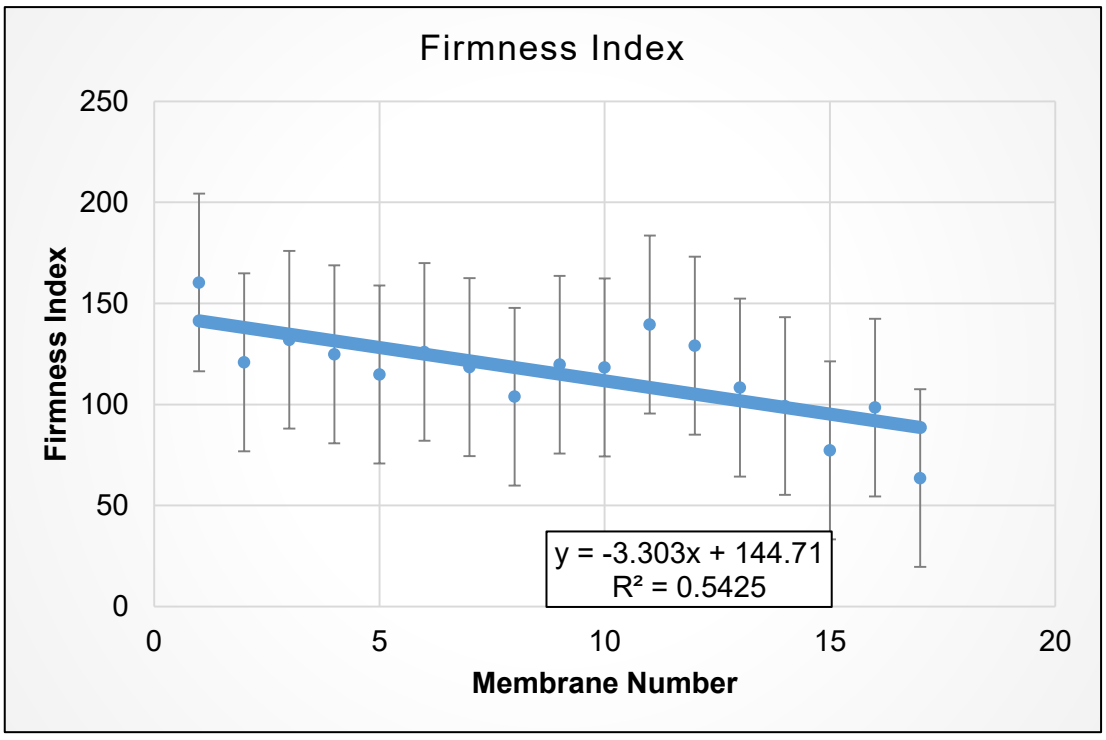

Figure 43. Correlation between mean FI values and membrane depth

From the graphs, it is possible to appreciate the trend of the two index in relation to the depth of the membrane. The viscoelasticity shows a direct

correlation with the membrane depth: the mean value for the membranes n. 1 is VI=61,78 while the mean value for membranes n.17 is VI=114,08, so the viscoelasticity of membranes shows an increase with depth. On the contrary, the firmness index values showed an inverse relationship with membrane depth: the more the depth the lower the firmness index of the membrane, with a mean value FI=160,34 for membranes n.1 and FI=63,57 for membranes n.17. While this correlations are far from perfect linearity, they give us a confirmation of the potential inverse relationship between the two parameters.

Moreover, understanding the relationships between the variables and among mechanical parameters and membranes position within the hoof could be critical to speed up sample selection process for a particular experiment.

As for other techniques consider in this chapter, mechanical evaluation could be of great use in selection of membranes to simulate a specific model of unhealthy nails, in particular in those cases where the pathology in directly related to the mechanical properties, such as brittle nail syndrome, but also psoriasis and onychomycosis. Based on VI and FI values membranes could be selected to mimic extreme situation on hard and soft nails.

Additionally, the assessment could be an in vitro safety study to be implemented for all products designed to be used on nails, to demonstrate the absence of adverse effects. Moreover, mechanical evaluation could be crucial for claim support.

CONCLUSION

The main goal of this part of the work was to evaluate and test a complete range of characterization techniques on hoof membranes as nail in vitro model, in order to assess the suitability of the model not only to be used for permeability studies but also as a model for in vitro evaluation of safety and efficacy of nail products.

The production method developed by our research group has shown to be effective, making it possible to obtain membranes with desirable thickness, homogenous within thickness group.

Hoof membranes have shown to be suitable for a wide range of surface characterization techniques. The results obtained suggest hoof membranes

suitability for assessment of both safety and efficacy of products willing to act on the nail plate. This research could be considered as a feasibility assessment for the use of solid surface analysis techniques in both characterization and product evaluation process. However, there is a huge potential to be explored: for example Z potential analysis has been applied for absorption study on surfaces of several nature in other field, and in addition to contact angle assessment it could be crucial in understanding and describing the interaction of a novel ingredient or product with nail.

Mechanical properties were the most challenging to be assessed, also for the poor coverage of the topic by scientific literature. Nail StrainStress meter NM100, a novel instrument already validated to be used in vivo, has shown to be a suitable instrument also for in vitro application. In fact, using hoof membranes for this particular assessment have shown both advantages and challenges. Membranes are of course still during analysis but challenging to be positioned correctly. However, the operation could then be totally standardized avoiding variability and out of control bias which could occur working with volunteers in in vivo analysis, without loosing the idea of simulation of real-life conditions. Mechanical properties of hoof membranes are not an exact transposition of the healthy human ones, instead they could be the perfect model to simulate unhealthy nails. The next step of the work will be in fact the application of this in vitro model for assessment of the efficacy of several nail products, with a particular focus on those addressing mechanical properties issue.

CHAPTER 2

PRODUCTS EFFICACY ASSAYS

INTRODUCTION

In this chapter of the thesis are presented materials, methods and results of different studies to assess efficacy of cosmetics products available on the market all supplied by Mavala®. All the claims have been already supported by the company through self-assessment in vivo studies on volunteers before reaching the market. The main goals of our studies are:

- To support the efficacy claims through objective studies;
- To evaluate the potentiality of our nail in vitro model and protocols to assess products efficacy.

In Table 19 there is a summary of the products tested, along with their main efficacy claims and the main characteristics of the protocol setup. All the protocols applied have been developed by our research group.

Table 19. List of Mavala products tested along with their specific claims and the main features of the protocol applied.

MAVALA Product	**Effect**	**Claim**	**Protocol Setup**	
			Membrane Properties	**Tested Parameters**
(PRODUCT A)	Fortifying and protective base coat for nails	- To reinforce the nail structure - To fortify the nail plate	Soft nail model	Firmness Index, Viscoelasticity index; Surface Improvements
(PRODUCT B)	Nail hardener	- To Harden the nail plate - To avoid breaking and splitting of the nail		
(PRODUCT C)	Moisturizing serum for dry and too hard nails	- To restore the flexibility to the nail plate and make it more resistant to external shocks	Brittle and fragile nails model	

		- To Moisturize the nail plate		
[redacted] **(PRODUCT D)**	Nutritive cream for damaged nails	- To restore the flexibility to the nail plate and make it more resistant to external shocks - To Moisturize the nail plate - To improve the general quality of the nail		
[redacted]	Base coat (water based)	- To prevent dehydration, splitting, flaking of nails - To form a protective barrier between the nail plate and nail coloured polish preventing yellowing of nails and improve adherence of nail polish.	- In vitro study with smooth membranes - In vivo study on volunteers ASSESSMENT through Colorimeter	
[redacted]	Base coat	- To isolate the nail plate from pigments, protecting it against yellowing - To extend manicure duration		

All the membranes have been prepared and characterized for these studies following procedures and techniques described in details in the previous chapter of this work.

In particular, all mechanical measurements reported were made in an air conditioned room with controlled temperature and humidity (T 25°C, R.H. 50 ± 5%) on cleaned and dried membranes; results of each parameter are expressed as mean results of at least 3 measurements on each membrane.
Statistical analyses was carried out on the results using a T-Test. A significance level of 5% was chosen, so the changes were considered

statistically significant for p <0.05. Other conventional symbols used are: not significant (n.s.) for p>0.05; slightly significant (*) for p≤0.05; significant (**) for p≤0.01; very significant (***) for p≤0.001.

[redacted] (PRODUCT A)

Materials and Methods

[redacted] PRODUCT A is described as a fortifying base which allows splitting and soft nails to regain their normal and strong aspect. It combines two actions working in synergy: fortifying and protecting. It is a colorless and shiny nail polish whose active ingredients combining technicality and efficiency to improve nails' quality and to reinforce their structure. Its complex of micro encapsulated active ingredients (tea tree essential oil, vitamin E, hydrolyzed keratin and arginine) fortifies nails and the crystal resin in it makes the nail plate more resistant and thicker.

This in vitro study can be divided in two part:

1. In Vitro Efficacy Evaluation;
2. Assay of the permeability of the active ingredients.

In Vitro Efficacy Evaluation

More than 100 membranes of about 300 µm thickness were obtained from bovine hooves and mounted on suitable PFTE custom made supports so that remain fixed throughout the experiment. In order to start with membranes with suitable and homogeneous properties, at the beginning of the study, in addition to the morphological evaluation, Thickness, Firmness (FI) and Viscoelasticity (VI) were measured by using the Nail StrainStress Meter NM100 (Courage & Khazaka, Cologne, Germany) described in chapter 1 of the present work. The same parameters have been used as endpoints at the end of the efficacy assay.

In particular, 54 membranes with flaky surface and a Viscoelasticity Index (VI) > 100 were selected as soft nail model and divided in three groups (18 membranes each group):

- Group 1: PLACEBO: OS22.21 – sans actives (formulation without actives);
- group 2: [redacted]-2 ACTIVES: 990.01
- group 3: [redacted]-3 ACTIVES: OS22.19

Tables 20-21-22 report mechanical data obtained from all membranes at the beginning of the experiment (SD%: percentage standard deviation; FI: firmness index; VI: viscoelasticity index).

Table 20. Mechanical data obtained from membranes from GROUP 1 at the beginning of the experiment (t0).

Subject	***VI***	***FI***	***Thickness(µm)***
1.13.10	178,22	172,81	272
1.15.9	112,12	104,07	337
1.6.11	152,60	98,36	298
1.9.12	155,20	116,67	285
2.12.15	141,36	57,23	368
2.7.11	142,55	99,25	296
3.7.7	135,87	130,86	343
3.9.6	137,94	153,14	315
4.10.9	111,58	106,13	317
4.7.5	150,92	105,11	388
4.9.11	110,27	114,02	304
4.9.14	128,25	91,57	356
A.11.15	139,98	52,21	264
A.11.3	156,65	141,05	206
A.3.3	115,52	82,87	309
A.8.4	121,26	146,14	313
B.10.10	129,18	78,87	350
B.10.4	116,21	185,71	294
Mean	**135,316**	**113,116**	**311,9**
SD%	**14,02**	**32,25**	**13,61**

Table 21. Mechanical data obtained from membranes from GROUP 2 at the beginning of the experiment (t0).

Subject	VI	FI	Thickness(μm)
1.13.11	123,22	110,82	317
1.2.7	169,39	163,95	264
1.6.9	119,11	90,39	358
1.9.7	169,37	160,00	253
2.12.5	110,15	117,88	299
2.7.5	117,64	135,42	330
3.9.11	182,46	118,87	294
3.9.8	144,91	94,30	293
4.13.8	136,07	94,33	311
4.8.9	173,55	105,85	318
4.9.12	127,59	116,49	287
4.9.7	101,65	145,18	339
A.11.19	103,45	131,15	308
A.11.5	175,66	175,00	229
A.3.7	101,20	101,01	359
A.8.6	157,72	139,33	312
B.10.15	166,69	114,31	290
B.10.5	126,69	89,80	323
Mean	**139,251**	**122,449**	**304,7**
SD%	**20,41**	**21,35**	**11,04**

Table 22. Mechanical data obtained from membranes from GROUP 3 at the beginning of the experiment (t0).

Subject	VI	FI	Thickness(μm)
1.15.6	117,51	93,97	303
1.2.8	123,49	91,31	314
1.8.5	107,51	186,35	301
2.10.10	153,76	98,50	266
2.6.3	102,80	86,70	331
3.11.16	119,98	185,74	328
3.9.16	146,81	69,49	351
4.10.10	100,97	170,32	298
4.15.4	129,96	93,89	244
4.9.10	119,86	88,21	329
4.9.13	120,21	81,75	309
4.9.9	135,89	149,96	281
A.11.2	191,11	159,31	199
A.11.7	187,42	43,49	341
A.8.10	112,13	95,94	312
A.8.8	170,52	134,85	270
B.10.3	101,98	143,68	311
B.10.8	159,61	76,61	301
Mean	**133,417**	**113,893**	**299,4**
SD%	**21,44**	**37,43**	**12,33**

The products were applied to the membranes every 72 hours for a total of 16 days with the products left on. The products were removed with a standard acetone based polish remover and before the next application the membranes were also washed with hands-soap, rinsed with warm water following a specific protocol and left drying in a conditioned room. After 16 days the membranes were cleaned one last time and then left to dry for at least 3 days before the instrumental evaluations (t1).

Permeability assay

The first step was to determine which component to use as a suitable analyte for quantification assay: we chose the water soluble amino acids such as which are all part of one of the active ingredient composition.

The analytical method selected for the quantification is reported in literature [95] [96]. It is based on the reaction of OPA (o-Phtalaldehyde) specifically with

primary amines in presence of thiols. We chose to evaluate sample absorbance (λ=340nm) using a spectrophotometer (UV-1900 Shimadzu).

The reference stock solutions were prepared using Threonine, Glutamic Acid and Arginine (Carlo Erba) in distilled water (1mg/ml); the dilutions were made in carbonate buffer (pH=10.5) as required by the assay. Active ingredient stock solution (10% v/v) has been prepared in distilled water and the following dilutions in carbonate buffer.

For the actual permeability assay we used a vertical Franz diffusion cell with fixed volume receptor chamber, controlled temperature, port to sample receptor fluid and stirred receptor fluid.

For the permeability assay more than 50 membranes of about 100-250 μm thickness were obtained from bovine hooves and characterized before the experiment.

Several experiments have been conducted using two different media in the acceptor compartment (water and carbonate buffer) and different concentration in the donor compartment of the diffusion cells. Every experiment has been performed in triplicate.

The best permeability results have been obtained setting the following parameters:

Table 23. Preferred parameters for the permeability study.

Time	24 hrs
Temperature	37°C
Stirring Rate	90 rpm
Donor	800 µl (1mg/ml)
Acceptor	Carbonate Buffer (5ml)

Contact Angle

Moreover, all study membranes were characterized using Contact Angle Meter DMe-211Plus (KYOWA). The contact angle is a measure of the wettability of a solid by a liquid [81], in this case membranes and water or carbonate buffer. For the efficacy assay, the measurements were taken with distilled water on 5 membranes for each group at first before the study, secondly with the product applied on and after the last removal. For the permeability assay, the contact angle measurement gives information on the compatibility of the membranes with the media used for the assay, and it has been measured for all the membranes used both with water and carbonate buffer.

Results and Discussion

In Vitro Efficacy Evaluation

In order to support [redacted] PRODUCT A commercial claims, the membranes were selected as a soft nail model (flaky or flaky/smooth surface and a VI <100).

Morphological Evaluation

In Figures 44-45-46 are reported some pictures where it is possible to compare the surface before the beginning of the study (t0) and after (t1) the last removal of the product.

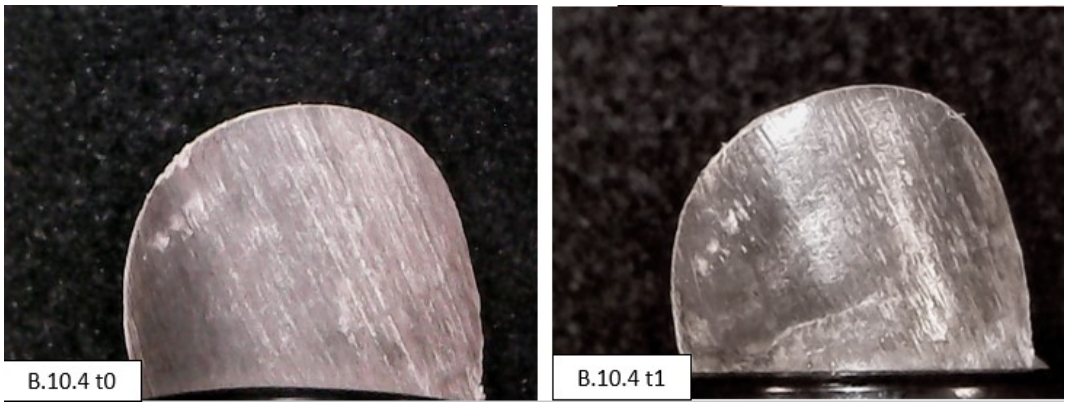

Figure 44. Membrane B.10.4 before the study (t0) and after (t1) the last removal of the PLACEBO nail polish (GROUP 1)

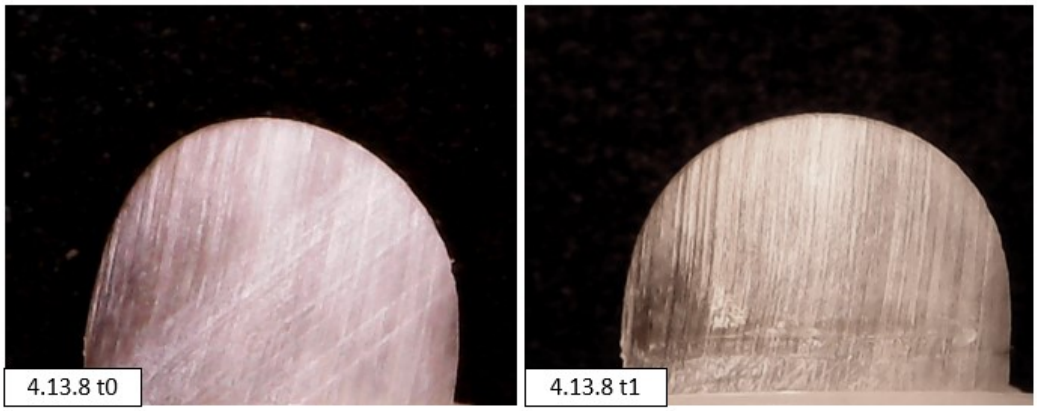

Figure 45. Membrane 4.13.8 before the study (t0) and after (t1) the last removal of the nail polish formulation with 2 actives (GROUP 2)

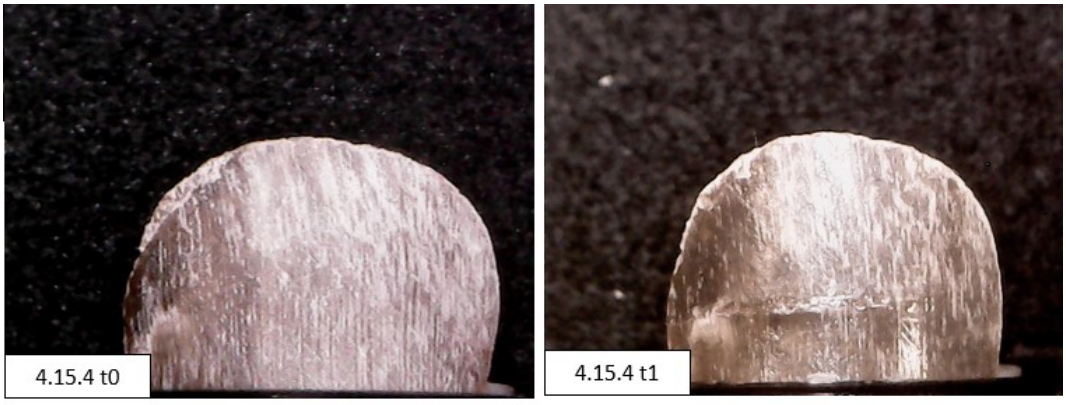

Figure 46. Membrane 4.15.4 before the study (t0) and after (t1) the last removal of the nail polish formulation with 3 actives (GROUP 3)

The membranes are smoother after the treatment, however, as it is clear from the examples above, even if the products have been removed with acetone

and the membranes have been washed after that, the polish is still visible on all the membranes independently from the group. This fact is proven also by the contact angle data.

In the table 24 are reported the mean percentage reduction of contact angle of the water drop on the membranes after 60 seconds from the deposition of the drop. The measurement have been taken before the beginning of the study (t0), after the application of the products on the membranes and after the last removal (t1).

Table 24. Percentage variation of the angle values over time in the three treatment groups

	VAR%		
	t0	post application	t1
Placebo	59,61	7,31	10,16
2 actives	68,89	7,34	7,58
3 actives	55,76	5,63	11,18

Before the treatment it is possible to appreciate the reduction of the angle over time, while after products application the angle values remain almost the same, showing only a small variation. The situation is almost unchanged after the last removal, which could be significant of the presence of a layer of actives, vehicle or both on the membranes but could be also due to a smoother surface.

Figure 47. Membrane 4.10.9 (placebo group) before the beginning of the study (t0). A: 0s after drop deposition; B: 30s; C: 60s

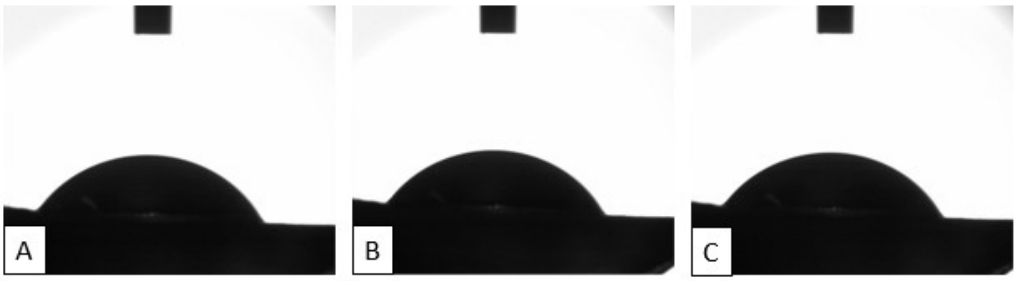

Figure 48. Membrane 4.10.9 (placebo group) after the application of the product. A: 0s after drop deposition; B: 30s; C: 60s

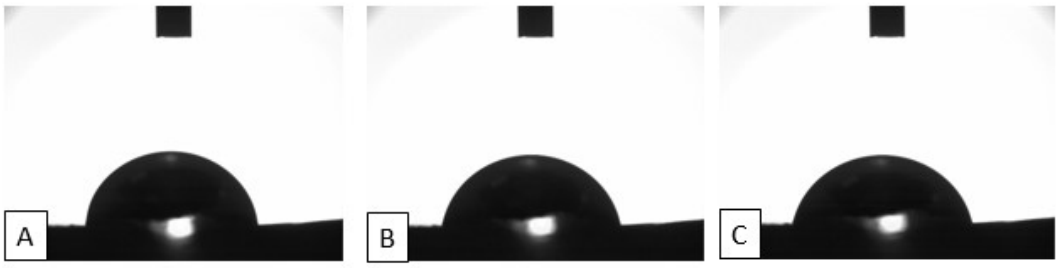

Figure 49. Membrane 4.10.9 (placebo group) at the end of the study (t1). A: 0s after drop deposition; B: 30s; C: 60s

Mechanical Evaluation

Here in the tables below is shown the comparison between the values obtained from the instrumental evaluation at the beginning and the end of the study.

Table 25. Mechanical data obtained from membranes fro Group 1 (Placebo treatment) at the beginning (t0) and at the end of the experiment (t1). Results are expressed as a mean of at least 3 measurements.

Membrane N.	*Vl_t0*	*Vl_t1*	*Fl_t0*	*Fl_t1*	*Thickness (µm) t0*	*Thickness (µm) t1*
1.13.10	178,22	182,42	172,81	116,14	272	285
1.15.9	112,12	66,56	104,07	105,86	337	490
1.6.11	152,60	105,39	98,36	77,12	298	378
1.9.12	155,20	127,79	116,67	97,85	285	400
2.12.15	141,36	100,02	57,23	95,82	368	382
2.7.11	142,55	94,49	99,25	82,87	296	381
3.7.7	135,87	90,51	130,86	156,83	343	400
3.9.6	137,94	126,36	153,14	124,06	315	344
4.10.9	111,58	91,22	106,13	114,76	317	365
4.7.5	150,92	87,28	105,11	105,34	388	507
4.9.11	110,27	111,07	114,02	121,62	304	358
4.9.14	128,25	95,10	91,57	85,86	356	376
A.11.15	139,98	127,92	52,21	59,32	264	390
A.11.3	156,65	136,78	141,05	126,81	206	283
A.3.3	115,52	107,65	82,87	83,84	309	371
A.8.4	121,26	70,91	146,14	130,50	313	432
B.10.10	129,18	87,83	78,87	98,84	350	438
B.10.4	116,21	115,47	185,71	165,51	294	336
Mean	**135,316**	**106,932**	**113,116**	**108,276**	**311,9**	**384,2**
SD%	**14,02**	**25,33**	**32,25**	**25,02**	**13,61**	**15,12**

Table 26. Mechanical data obtained from membranes Group 2 (2 Active ingredients) at the beginning (t0) and at the end of the experiment (t1). Results are expressed as a mean of at least 3 measurements.

Membrane N.	*Vl_t0*	*Vl_t1*	*Fl_t0*	*Fl_t1*	*Thickness (µm) t0*	*Thickness (µm) t1*
1.13.11	123,22	91,24	110,82	127,34	317	414
1.2.7	169,39	126,58	163,95	221,16	264	319
1.6.9	119,11	85,08	90,39	135,41	358	444
1.9.7	169,37	87,25	160,00	158,09	253	393
2.12.5	110,15	92,44	117,88	135,64	299	305
2.7.5	117,64	94,81	135,42	234,14	330	333
3.9.11	182,46	176,35	118,87	136,53	294	297
3.9.8	144,91	116,46	94,30	134,62	293	328
4.13.8	136,07	96,14	94,33	214,15	311	321
4.8.9	173,55	97,41	105,85	172,09	318	450
4.9.12	127,59	78,99	116,49	179,52	287	295
4.9.7	101,65	54,34	145,18	182,90	339	419
A.11.19	103,45	71,05	131,15	221,13	308	362
A.11.5	175,66	83,62	175,00	290,07	229	330
A.3.7	101,20	93,44	101,01	137,90	359	390
A.8.6	157,72	105,97	139,33	149,90	312	450
B.10.15	166,69	142,32	114,31	155,88	290	390
B.10.5	126,69	93,11	89,80	279,90	323	369
Mean	**139,251**	**99,256**	**122,449**	**181,466**	**304,7**	**367,2**
SD%	**20,41**	**27,77**	**21,35**	**28,04**	**11,04**	**14,59**

Table 27. Mechanical data obtained from membranes fro Group 3 (3 Active ingredients) at the beginning (t0) and at the end of the experiment (t1). Results are expressed as a mean of at least 3 measurements.

Membrane N.	*VI_t0*	*VI_t1*	*FI_t0*	*FI_t1*	*Thickness (µm) t0*	*Thickness (µm) t1*
1.15.6	117,51	103,65	93,97	143,42	303	324
1.2.8	123,49	107,01	91,31	163,34	314	320
1.8.5	107,51	98,69	186,35	326,02	301	310
2.10.10	153,76	105,94	98,50	221,92	266	318
2.6.3	102,80	88,51	86,70	105,59	331	361
3.11.16	119,98	82,07	185,74	223,27	328	328
3.9.16	146,81	117,70	69,49	115,81	351	330
4.10.10	100,97	94,05	170,32	112,90	298	365
4.15.4	129,96	97,40	93,89	144,59	244	262
4.9.10	119,86	74,49	88,21	207,94	329	344
4.9.13	120,21	69,83	81,75	100,59	309	356
4.9.9	135,89	100,20	149,96	251,88	281	365
A.11.2	191,11	135,34	159,31	230,07	199	224
A.11.7	187,42	62,35	43,49	88,94	341	374
A.8.10	112,13	87,54	95,94	151,44	312	363
A.8.8	170,52	76,38	134,85	215,54	270	321
B.10.3	101,98	81,19	143,68	96,93	311	347
B.10.8	159,61	106,39	76,61	128,92	301	326
Mean	**133,417**	**93,818**	**113,893**	**168,285**	**299,4**	**329,9**
SD%	**21,44**	**19,27**	**37,43**	**39,25**	**12,33**	**11,45**

Herewith below the graph of the mean viscoelasticity index over time for each groups is reported.

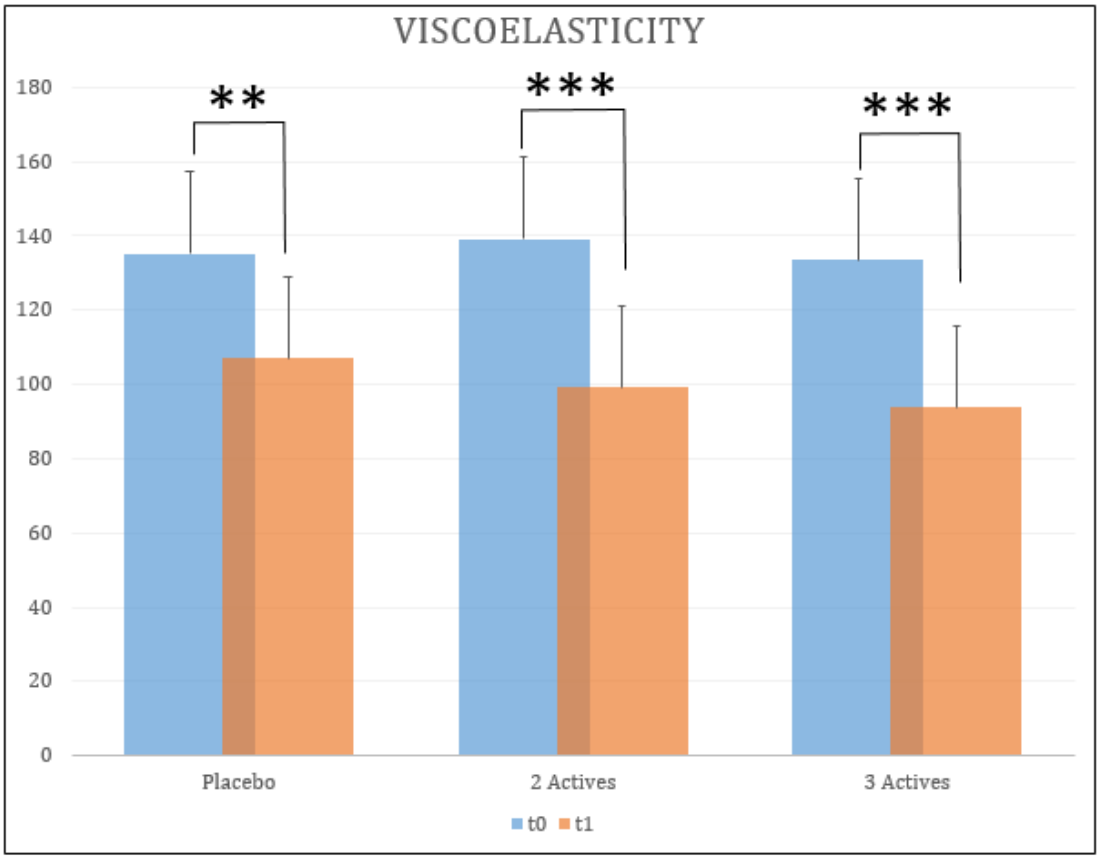

Figure 50. Mean Viscoelasticity index at the beginning (t0) and at the end of the study (t1)

The statistical analysis carried out on the samples showed a significant reduction of the viscoelasticity index of the membranes in all groups after the treatments (group 1: p=0,001**; group 2: p=0,0001***; group 3: p=2,8E-05***). Even if the reduction is more significant after treatment with 2 actives formulation and 3 actives formulation in comparison to the placebo group, no statistical significance has been found between these groups (t1 group 1 vs. t1 group 2: p=0,41; t1 group 1 vs. t1 group 3: p=0,10). A decrease in the viscoelasticity index could be translated in an increased stiffness of the membranes which was predictable considering the residual nail polish on the surface, but it was also desired as an effect of treatment. In fact, observing that the decrease is more important in group 2 and 3 may be due to the presence of the actives within the formulations.

Herewith below the graphs of the mean firmness index and mean thickness values over time are reported.

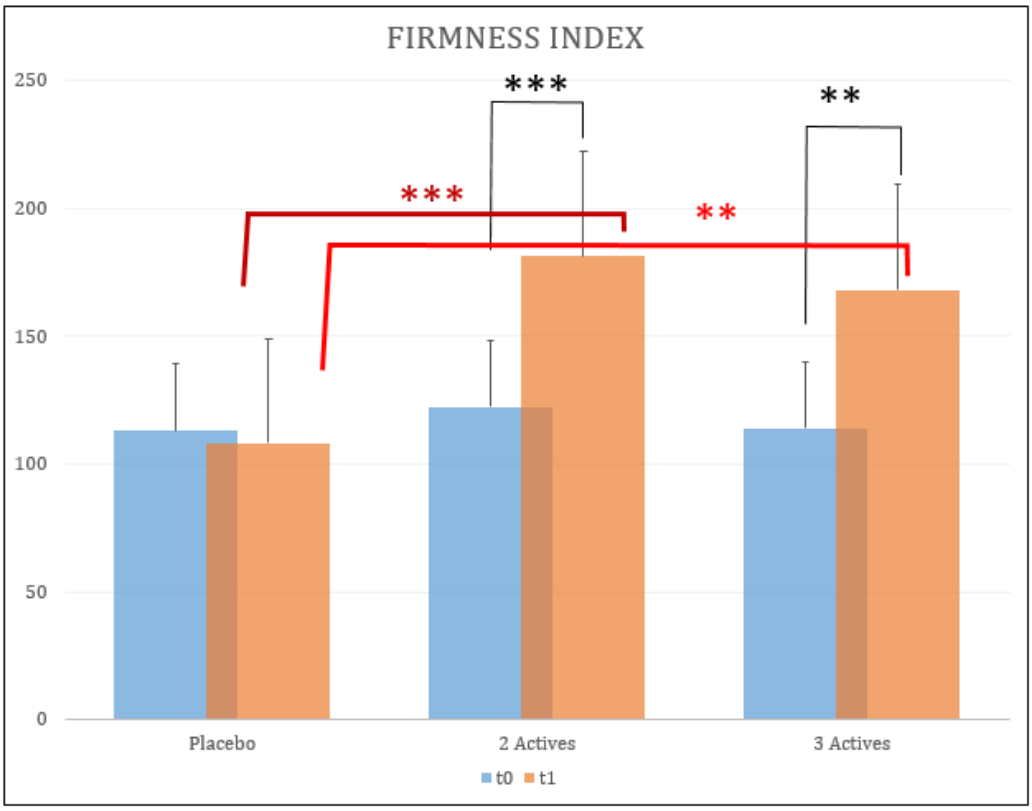

Figure 51. Mean Firmness index at the beginning (t0) and at the end of the study (t1)

The statistical analysis carried out on the samples showed a significant difference in the firmness index of the membranes of group 2 and group 3 at t1 in comparison to the data obtained on the same membranes at the beginning (group 2: p=0,0002***; group 3: p= 0,006**), while there is no difference between t0 and t1 data of placebo group (p=0,65).

The most interesting data is the statistical significant difference of firmness index data after the treatment (t1) between placebo and group 2 (p=1,22E-05***) and between placebo and group 3 (p=0,002**). These data came in support of the reinforce action claimed by the product: while with the viscoelasticity index it was not possible to see a statistical difference between placebo and active formulations, with the firmness index the effect of the active ingredients in comparison to the placebo is clear.

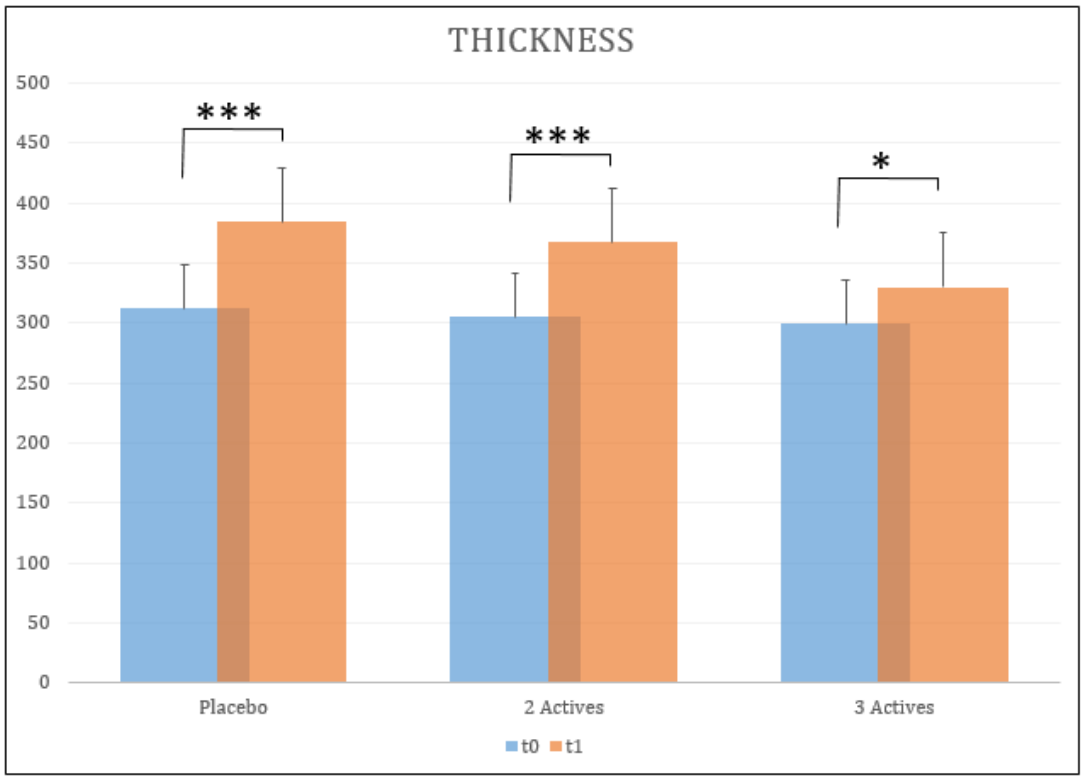

Figure 52. Mean Thickness value at the beginning (t0) and at the end of the study (t1)

The data shows an increase in membranes thickness in all groups (group 1: p=0,0002***; group 2: p=0,0002***; group 3: p=0,02*). This is a confirmation of the fact that a layer of product has remained attached to the membranes, however the increase observed is less significant for membranes of group 3. This could mean that the decreased found in the viscoelasticity index may be due to the active formulation itself rather than to residual nail polish.

Overall, the variations of the mechanical parameters are summarized in the Figure 53, which represents the mean of the % variation of the samples for each parameters.

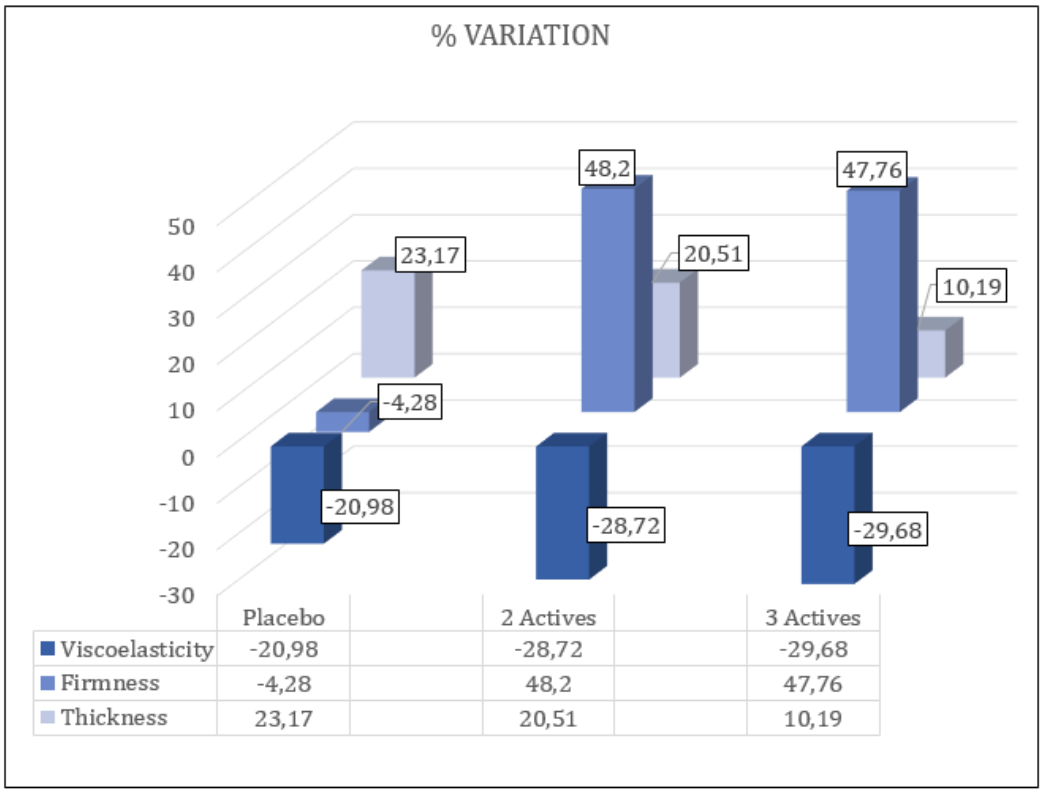

Figure 53. Mean % variation of each parameter over time

Permeability Evaluation

Analytical Assay

As reported in the materials and methods section, pure amino acids have been used as standard reference since they are contained in one of the active component of the final product.

All the blank and control analysis have been performed showing no interferences. Moreover, stability test has been performed to assess the evidence of degradation of the samples over time or by temperature (37°C) and in all cases the absorbance has been shown not be affected.

The graphs in figures 54-55-56 feature the relation between absorbance vale and the amino acid concentration and the linear regression equations along with the determination coefficient values (R^2) obtained.

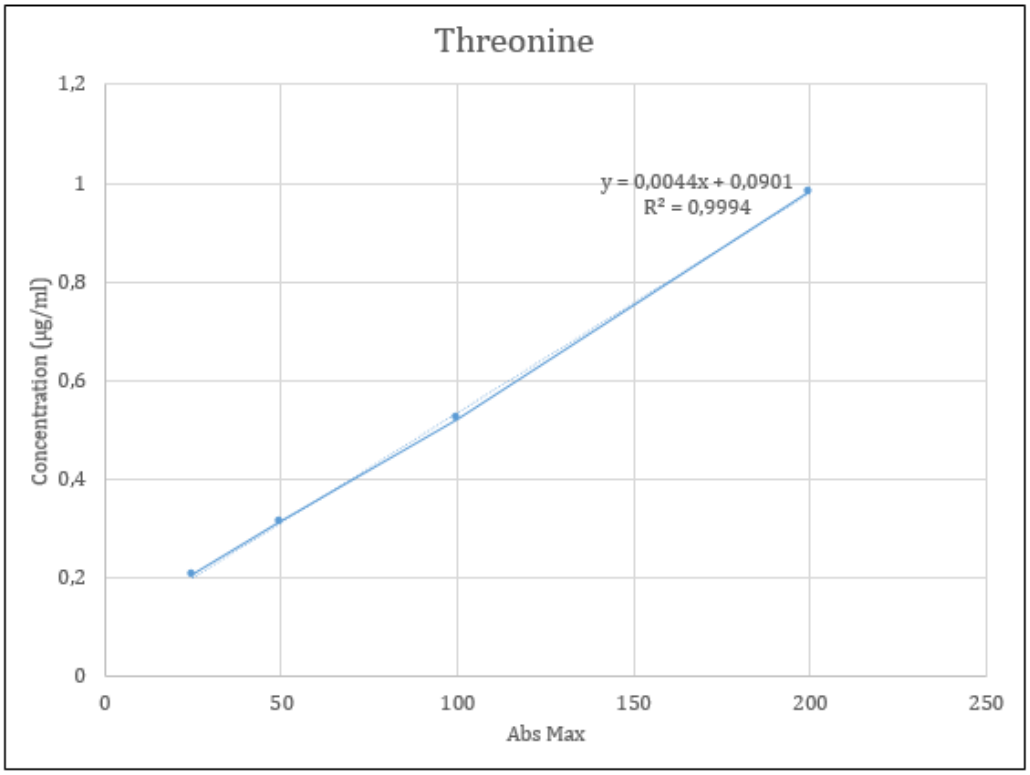

Figure 54. Threonine calibration curve and linear regression model (R^2=0,9994)

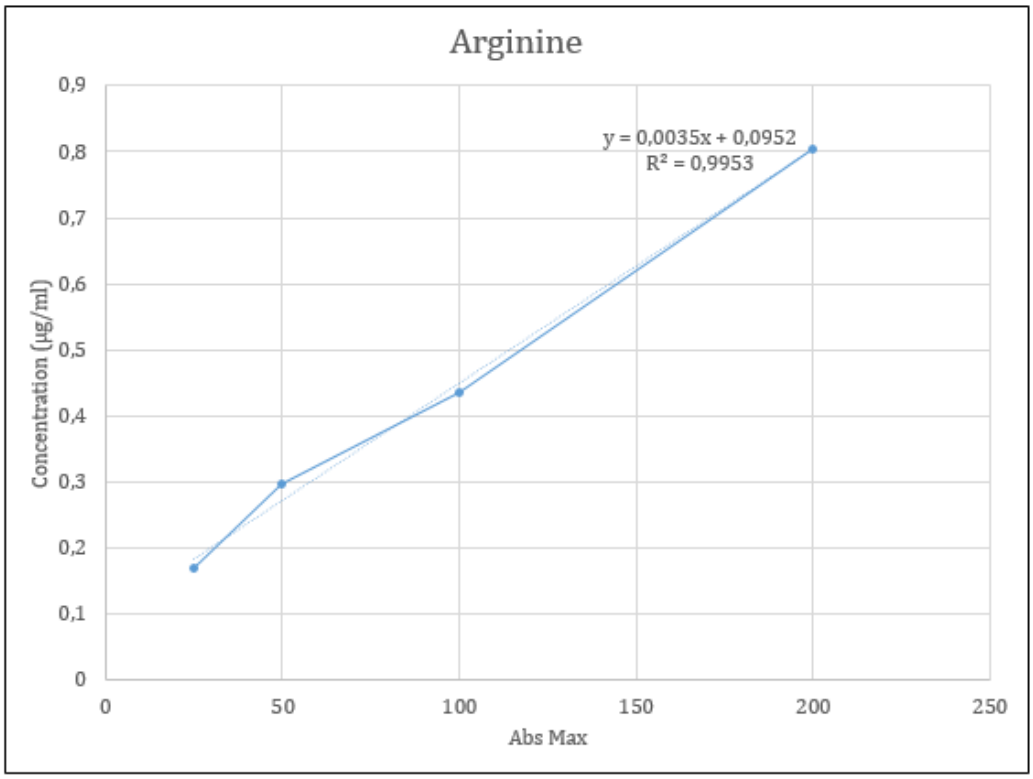

Figure 55. Arginine calibration curve and linear regression model (R^2=0,9953)

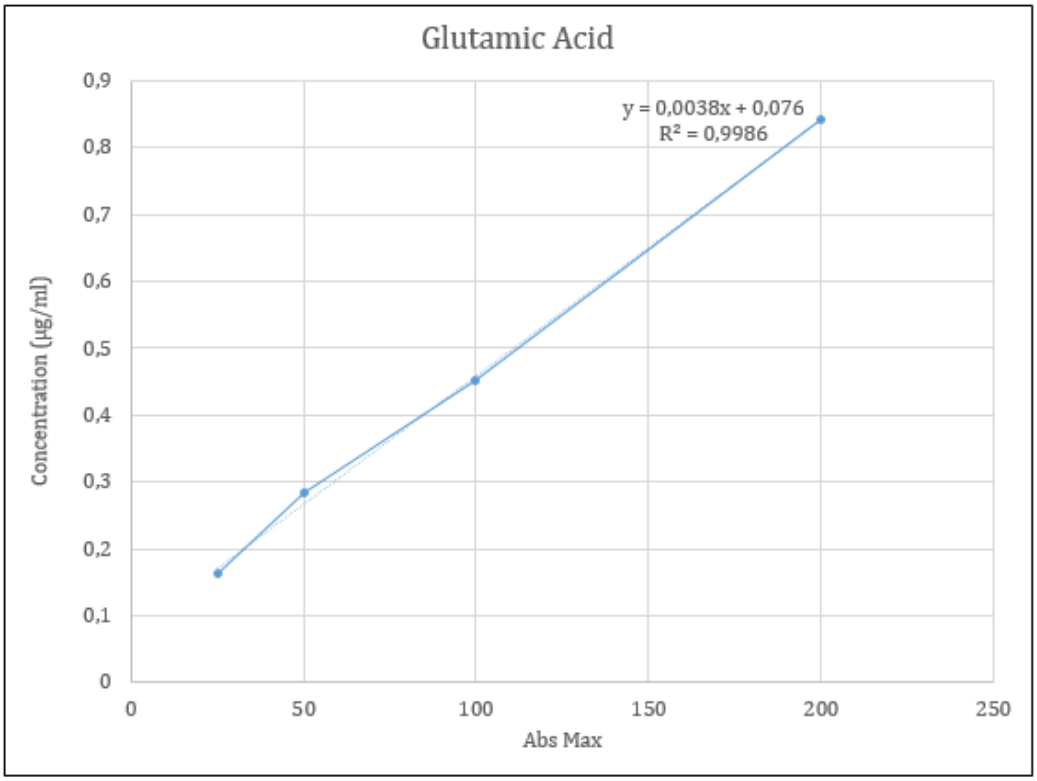

Figure 56. Glutamic Acid calibration curve and linear regression model (R^2=0,9986)

All the standards used has shown a good correlation between absorbance and concentration.

Once it has been established the linear trend of the standards, the assay with the active itself has been performed, both in water and in carbonate buffer, with no different results.

In figure 57 are reported the absorbance values obtained for the active ingredient dilutions in carbonate buffer in correlation with the concentration of the active, which has shown a perfect linearity (R^2=1).

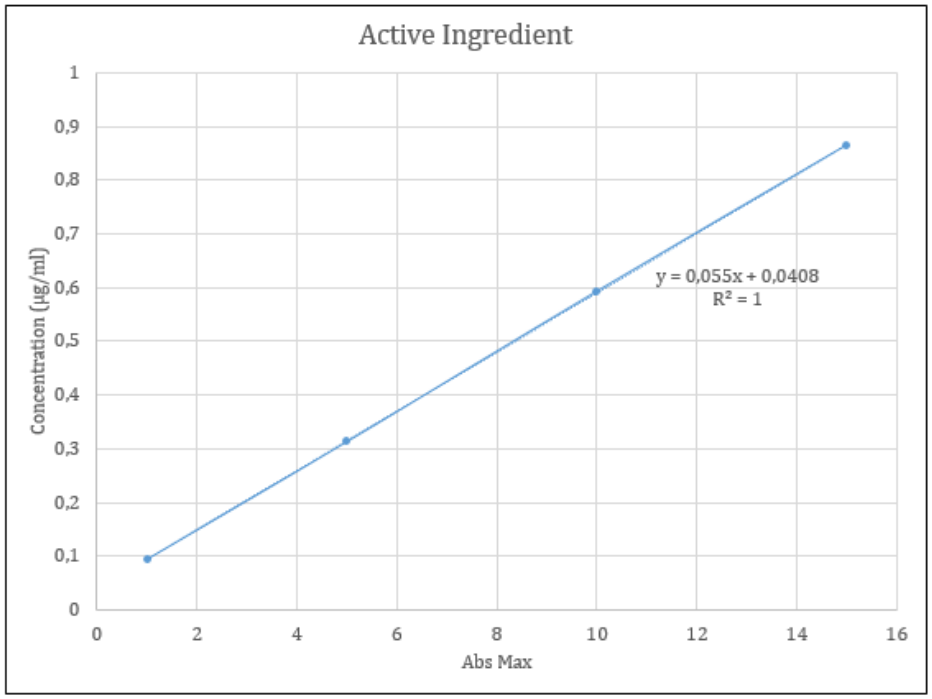

Figure 57. Active ingredient (buffer dilutions) calibration curve and linear regression model (R^2=1)

However, when performing the control analysis with only the sample of the active ingredient without the OPA reagent, it is still possible to detect an absorbance value, in contrast to the control with the amino acid standard solutions for which the absorbance value was zero when analysed alone. This is significant of the fact that the absorbance value obtained for the active ingredient is not only due to the presence of amino groups in the solution but it is the sum results of other factors too, such as turbidity.

To understand the contribution of the amino groups to the absorbance values (Abs Total) we subtracted from those values the ones obtained with the active ingredient dilution analysed alone (Abs Control), meaning without OPA reagent. Using the Threonine calibration curve as a reference of single amino group molecule, it is possible to calculate the amino group concentration and correlate it to the active ingredient concentration.

Table 28. Calculation of amino group concentration within Active ingredient

Active ingredient (mg/ml)	Abs Total	Abs Control	Difference	Conc (µg/ml) amino groups
5	0,314	0,13	0,184	21,34
10	0,593	0,272	0,321	52,48
15	0,864	0,439	0,425	76,11

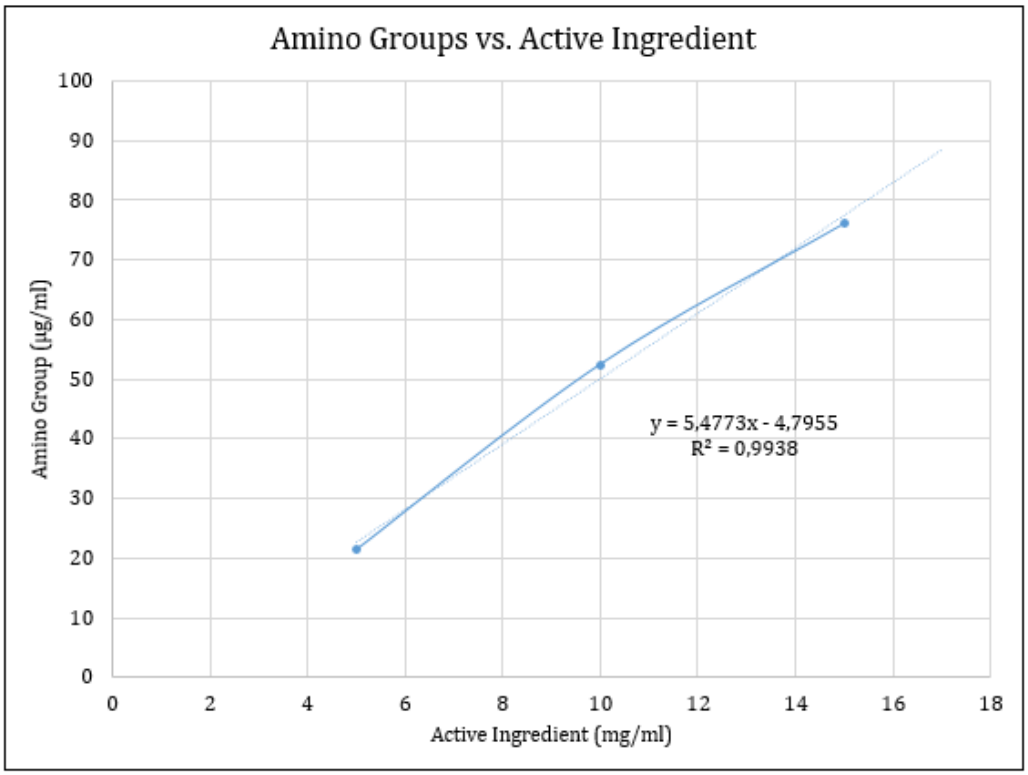

Figure 58. Amino groups as a function of Active ingredient concentration

Permeability Assay

In the table below are reported the membranes characterized for the permeability assays and their properties. Along with the membrane code is reported the thickness measurement and the classification based on surface morphology (F= Flaky; F/S= semi-flaky; S=smooth).

Table 29. Membranes selected for permeability assays

Membrane Code	Thickness (µm)	Morphology
A.6.3	150-230	F
A.6.4	220-290	F
A.6.7	140-370	F
A.6.9	150-170	F
A.6.10	180-300	F
A.6.11	180-270	F
A.6.13	160-270	F/S
A.6.14	170-260	F/S
A.1.5	270-300	F
A.9.8	220-240	F
A.9.20	220-240	F/S
1	110-150	S
2	220-270	F
3	160-180	F
4	120-140	F
5	150-210	F
6	110-130	S
7	120-150	F/S
8	80-100	F/S
9	110-120	S
10	130-150	S
11	120-140	F/S
12	100-130	S

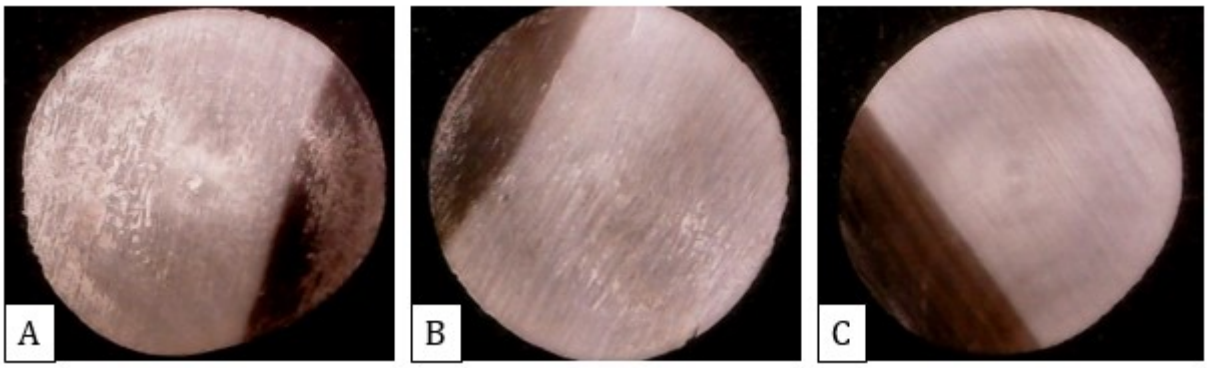

Figure 59. Examples of Flaky (A), Semi-Flaky (B) and Smooth (C) surface

All the membranes have been characterized also for their wettability using Contact Angle Meter DMe-211Plus (KYOWA), both with water and carbonate buffer. While there is variability with water wettability, all the membranes have shown high wettability with carbonate buffer with and angle reduction over time between 80% and 100%.

The best permeability results have been obtained setting the following parameters:

Table 30. Summary of parameters applied for permeability assay.

Time	24 hrs
Temperature	37°C
Stirring Rate	90 rpm
Donor	800 µl (1mg/ml)
Acceptor	Carbonate Buffer (5ml)

After 24 hours, the solution in the acceptor compartment has been sampled and analysed and using threonine calibration curve equation (y=0,0044x + 0,0901) the concentration of the threonine in the compartment has been calculated. In the table below are reported the data obtained and the calculation of the percentage of threonine that permeate the membranes in 24 hrs (highlighted in yellow).

Table 31. Threonine permeability assay results.

Sample	Abs	Conc (µg/ml)	µg in 5ml	%
1	0,204	25,89	129,43	16,18
A.6.4	0,182	20,89	104,43	13,05
A.6.10	0,215	28,39	141,93	17,74
Mean	**0,200**	**25,05**	**125,27**	**15,66**

In addition to the solution in the acceptor compartment, after the 24 hours, also the remaining solution in the donor compartment has been analysed and whatever has been absorbed on the membranes has been extracted. Using the same calculation method as before it was possible to calculate the percentage of threonine in every solution in comparison to the total amount put in the donor compartment at the beginning.

Table 32. Percentage of threonine in donor compartment and extracted from the membranes after 24 hours

Sample	% Donor (24 hrs)	% Membrane (24 hrs)
1	56,58	/
A.6.4	/	9,20
A.6.10	44,24	/

The same experiment has been performed to assess Active ingredient permeability, using a 10% solution of Active ingredient in the donor compartment. The setting parameters are summarized in the table below.

Table 33. Summary of setting parameters for permeability assay

Time	24 hrs
Temperature	37°C
Stirring Rate	90 rpm
Donor	800 µl (100mg/ml)
Acceptor	Carbonate Buffer (5ml)

After 24 hours, the solution in the acceptor compartments has been sampled and analysed and using Active ingredient calibration curve equation

(y=0,055x+0,0408) the concentration of the Active ingredient in the compartment has been calculated. In the table below are reported the data obtained and the calculation of the percentage of active that permeate the membranes in 24 hrs.

Table 34. Active ingredient permeability assay results

Sample	Abs	Conc (mg/ml)	mg in 5 ml	%
A.1.5	0,323	5,13	25,65	25,65
A.9.20	0,23	3,44	17,20	17,20
A.9.8	0,256	3,91	19,56	19,56
Mean	**0,270**	**4,16**	**20,81**	**20,81**

Table 35. Percentage of Active ingredient in donor compartment and extracted from the membranes after 24 hours

Sample	% Donor (24 hrs)	% Membrane (24 hrs)
A.1.5	74,60	2,88
A.9.20	58,65	6,12
A.9.8	38,39	1,53

Using the equation obtained through the correlation between amino group and Active ingredient concentration (Figure 58. Eq: y=5,4773x-4,7955) it is possible to derive the amount of amino group permeated through the membranes in 24 hours. The results are shown in the table below and are consistent to the results obtained with the threonine alone.

Table 36. Percentage of Amino Group permeated through the membrane in 24 hours

Sample	% Amino Group (24 hrs)
A.1.5	20,45
A.9.20	12,32
A.9.8	14,59
Mean	**15,79**

To summarize, the first aim of this work was to evaluate the “in vitro” efficacy of [redacted] PRODUCT A to strengthen and restore the nail plate of soft and flaky nails. After 16 days of treatment on bovine membranes following the protocol described in this work, it is clear that the product induces an improvement in the surface appearance, making it more smooth and homogenous.

In particular, while viscoelasticity index and thickness have a similar trend in all groups, the significant increase in the firmness index of group 2 and 3 in comparison to the placebo group is a strong signal of the actives effect on the membranes properties. The next step would be to confirm the data through an in vivo study involving the mechanical assessment on the nails.

The second aim of the study was to evaluate the permeation properties of the product active components. We were able to demonstrate the permeation of one of the active ingredient (Mean=20,81%) and its aminoacidic components (Mean=13,72%) through the nail barrier over time (24 hrs).

Moreover, 2-6 % of the active has been extracted from the membranes after 24 hours, which could possibly be significant of the affinity of the active for the membranes and a confirmation of the data obtained with the in vitro efficacy assay. While these results are promising and the protocol seem to be reliable, a higher number of replies would allow a more solid evaluation of the data through a statistical analysis.

[redacted] (PRODUCT B)

Materials and Methods

[redacted] PRODUCT B claims to harden the nail plate thanks to a scientific active ingredient that should improve the cohesion of keratin fibres of the the three principal layers of the nail plate. It also contains crystal resin tears, a gum extracted from the pistachio mastic tree that helps the natural keratinization process of nails, reinforcing them. From a technological point of view, it is aqueous solution, that is neither a base nor a nail polish, thus it does not require nail polish remover. In order to support the claims, a specific in vitro protocol has been developed using membranes obtained from bovine hooves with the suitable mechanical properties (soft nails model) for the product application. In particular, 58 membranes with flaky surface, a Firmness Index < 130 and a Viscoelasticity > 74 were selected and divided in two groups:

1. CONTROL group (28 membranes): wash procedure only
2. PRODUCT group (30 membranes) : [redacted] 089150/ 995.01

Tables 37-38 report mechanical data obtained from all membranes at the beginning of the experiment. Results are expressed as a mean of at least 3 measurements (SD%: percentage standard deviation; FI: firmness index; VI: viscoelasticity index).

Table 37. Mechanical data obtained from membranes from CONTROL group at the beginning of the experiment (t0). Results are expressed as a mean of at least 3 measurements.

Subject	VI	FI	Thickness
1.15.6	114,82	97,88	313
1.6.18	107,50	120,26	292
2.10.15	103,89	81,47	330
2.10.8	100,30	69,47	336
2.12.5	80,72	94,25	328
2.3.5	99,68	61,39	346
2.6.5	203,25	90,04	288
2.7.8	124,02	90,03	317
2.8.5	79,46	107,65	317
2.9.3	182,41	102,59	258
2.9.9	115,96	46,32	358
4.17.5	76,41	94,93	338
2.12.15	244,45	66,45	313
2.8.10	106,66	93,60	274
2.9.5	104,41	67,07	331
3.7.3	130,93	74,99	310
3.9.10	165,77	66,15	282
3.9.16	233,84	95,71	313
4.10.12	137,49	85,11	277
4.10.8	115,39	85,89	326
4.13.6	61,09	79,61	295
4.15.7	122,52	71,61	279
4.8.15	86,16	72,88	297
4.8.6	90,65	118,69	381
4.8.9	145,86	116,35	279
2.12.9	84,03	89,08	330
3.7.4	79,56	118,26	280
4.8.10	90,74	63,62	276
Mean	**121,000**	**86,477**	**309,4**
SD%	**38,47**	**22,34**	**9,45**

Table 38. Mechanical data obtained from membranes from PRODUCT group at the beginning of the experiment (t0). Results are expressed as a mean of at least 3 measurements.

Subject	VI	FI	Thickness
1.13.5	82,40	118,87	351
1.2.5	103,83	93,52	244
1.7.5	129,50	101,21	311
1.7.6	114,67	118,49	310
1.7.8	131,81	76,49	322
1.7.9	164,55	45,35	332
1.8.10	77,06	80,30	346
1.8.9	107,12	97,47	362
2.12.6	84,99	99,12	375
2.6.8	79,09	101,54	332
2.7.4	98,11	112,42	303
2.8.7	138,51	47,64	368
2.9.6	84,23	114,31	284
2.9.8	167,89	79,85	324
3.11.10	78,01	95,15	326
3.11.12	93,48	126,31	342
3.11.13	127,29	90,56	337
3.11.14	155,85	107,91	314
3.11.4	102,22	121,67	303
3.11.5	98,49	82,96	335
3.11.8	116,90	101,12	307
3.9.13	81,23	83,33	321
3.9.15	123,51	96,37	284
4.13.7	77,26	116,69	317
4.15.6	91,48	112,52	322
4.5.3	214,72	108,25	237
4.7.10	193,53	122,91	307
4.7.3	90,39	101,00	428
4.7.4	129,27	73,88	393
4.7.8	115,65	83,92	294
Mean	**116,343**	**96,883**	**324,1**
SD%	**30,50**	**21,28**	**12,16**

The product was applied to the membranes of the product group every 24 hours with the same brush; before each application membranes were cleaned with hands-soap, rinsed with warm water following a specific protocol and left drying in a conditioned room. The membranes of the control group were subjected to the cleaning procedure only. After 14 applications the membranes were rinsed one last time and then left to dry for at least 3 days before the instrumental evaluations (t1).

Results and Discussion

Morphological Evaluation

The membranes has been selected for the study with a flaky surface, to evaluate the effects of the product on the morphology of a damaged nail. Here below are reported some pictures, where it is possible to compare the surface before (t0) and after (t1) the application of the product.

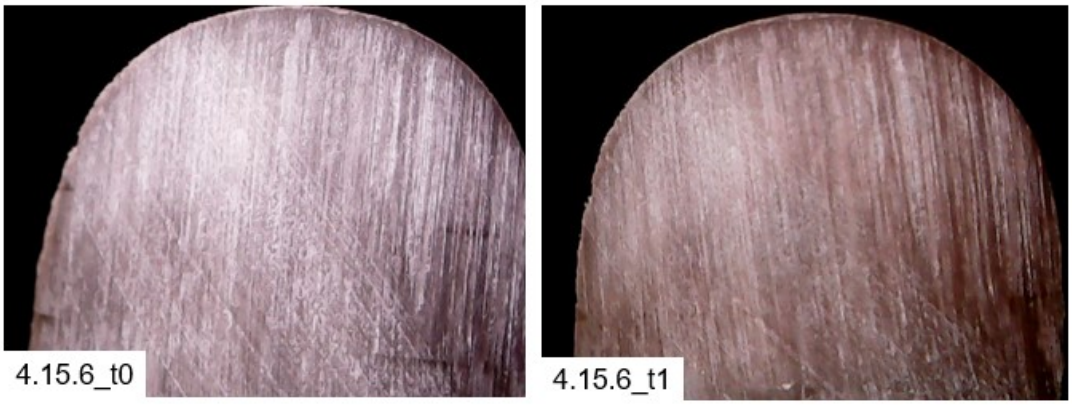

Figure 60. Membrane 4.15.6 before (t0) and after (t1) the treatment

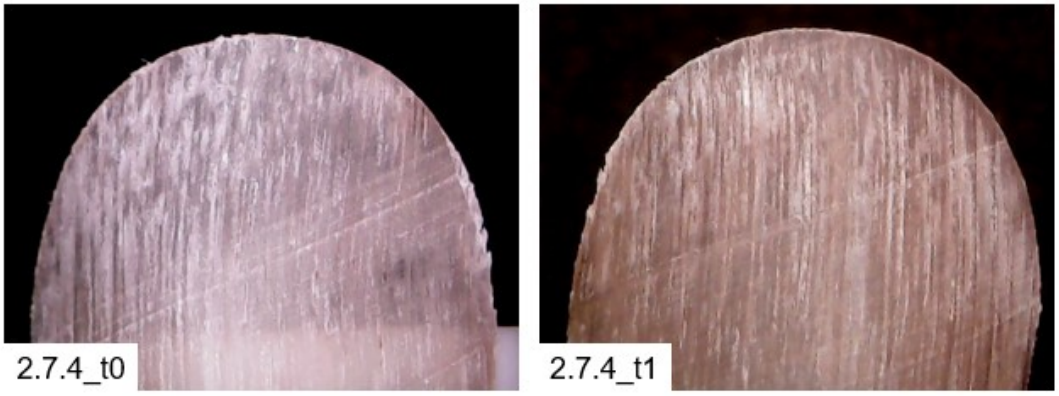

Figure 61. Membrane 2.7.4 before (t0) and after (t1) the treatment

Even if by pictures is not easy to appreciate the changes, the surface of the membranes appear more smooth and uniform in comparison the condition at the beginning of the experiment.

Mechanical Evaluation

Here in the tables below is shown the comparison between the values obtained from the instrumental evaluation at the beginning and the end of the study.

Table 39. Mechanical data obtained from membranes from control group at the beginning (t0) and at the end of the experiment (t1). Results are expressed as a mean of at least 3 measurements.

Membrane N.	*Vl_t0*	*Vl_t1*	*Fl_t0*	*Fl_t1*	*Thickness (µm) t0*	*Thickness (µm) t1*
1.15.6	114,82	141,79	97,88	46,79	313	319
1.6.18	107,50	161,99	120,26	185,03	292	259
2.10.15	103,89	144,78	81,47	101,55	330	313
2.10.8	100,30	123,19	69,47	143,87	336	285
2.12.5	80,72	115,80	94,25	215,30	328	280
2.3.5	99,68	97,85	61,39	81,62	346	319
2.6.5	203,25	151,49	90,04	96,64	288	246
2.7.8	124,02	119,58	90,03	90,15	317	260
2.8.5	79,46	100,10	107,65	100,37	317	296
2.9.3	182,41	226,66	102,59	186,46	258	207
2.9.9	115,96	183,25	46,32	87,59	358	277
4.17.5	76,41	83,82	94,93	158,54	338	292
2.12.15	244,45	159,85	66,45	55,34	313	371
2.8.10	106,66	112,30	93,60	127,10	274	301
2.9.5	104,41	84,92	67,07	96,89	331	332
3.7.3	130,93	138,97	74,99	58,12	310	360
3.9.10	165,77	154,94	66,15	83,17	282	304
3.9.16	233,84	129,81	95,71	45,23	313	401
4.10.12	137,49	171,99	85,11	77,40	277	317
4.10.8	115,39	98,27	85,89	105,58	326	313
4.13.6	61,09	66,55	79,61	122,05	295	317
4.15.7	122,52	117,42	71,61	97,81	279	312

4.8.15	86,16	93,09	72,88	141,80	297	306
4.8.6	90,65	78,75	118,69	127,26	381	385
4.8.9	145,86	137,50	116,35	179,28	279	311
2.12.9	84,03	95,13	89,08	123,21	330	312
3.7.4	79,56	168,97	118,26	84,14	280	337
4.8.10	90,74	199,65	63,62	226,05	276	269
Mean	**121,000**	**130,658**	**86,477**	**115,869**	**309,4**	**307,2**
SD%	**38,47**	**29,81**	**22,34**	**42,02**	**9,45**	**13,46**

Table 40. Mechanical data obtained from membranes from product group at the beginning (t0) and at the end of the experiment (t1). Results are expressed as a mean of at least 3 measurements.

Membrane N.	*Vl_ t0*	*Vl_t1*	*Fl_t0*	*Fl_t1*	*µm_t0*	*µm_t1*
1.13.5	82,40	86,46	118,87	126,89	351	351
1.2.5	103,83	211,65	93,52	100,45	244	265
1.7.5	129,50	127,49	101,21	96,10	311	307
1.7.6	114,67	84,02	118,49	83,53	310	344
1.7.8	131,81	124,99	76,49	97,20	322	323
1.7.9	164,55	157,07	45,35	68,69	332	346
1.8.10	77,06	153,84	80,30	102,14	346	299
1.8.9	107,12	153,22	97,47	117,47	362	337
2.12.6	84,99	136,61	99,12	129,05	375	353
2.7.4	98,11	127,19	112,42	132,59	303	319
2.8.7	138,51	178,28	47,64	168,44	368	307
2.9.6	84,23	207,80	114,31	160,65	284	303
2.9.8	167,89	89,85	79,85	94,37	324	322
3.11.10	78,01	105,42	95,15	117,96	326	305
3.11.12	93,48	188,84	126,31	160,01	342	315
3.11.13	127,29	112,06	90,56	182,45	337	331
3.11.14	155,85	175,91	107,91	94,28	314	343

3.11.4	102,22	200,14	121,67	167,27	303	313
3.11.5	98,49	161,15	82,96	100,48	335	340
3.11.8	116,90	88,13	101,12	76,38	307	338
3.9.13	81,23	90,30	83,33	59,52	321	376
3.9.15	123,51	144,76	96,37	148,31	284	295
4.13.7	77,26	113,80	116,69	72,03	317	342
4.15.6	91,48	124,13	112,52	123,80	322	322
4.5.3	214,72	142,27	108,25	166,70	237	230
4.7.10	193,53	169,69	122,91	68,28	307	347
4.7.3	90,39	79,55	101,00	109,30	428	474
4.7.4	129,27	60,85	73,88	107,56	393	387
4.7.8	115,65	133,68	83,92	114,32	294	312
Mean	**116,343**	**135,487**	**96,883**	**115,387**	**324,1**	**329,2**
SD%	**30,50**	**30,24**	**21,18**	**29,62**	**12,16**	**12,61**

Herewith below the graph of the mean viscoelasticity index over time for both groups is reported.

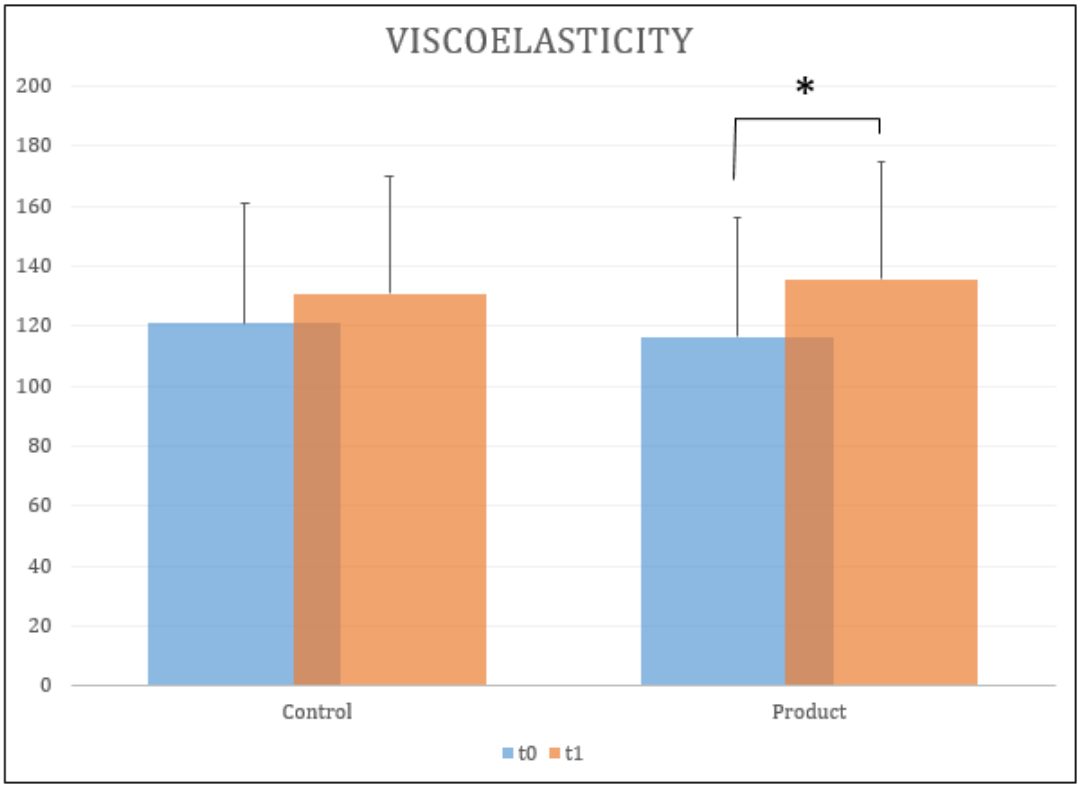

Figure 62. Mean Viscoelasticity index at the beginning (t0) and at the end of the study (t1)

The statistical analysis carried out on the samples showed a significant difference in the viscoelasticity index of the membranes treated with PRODUCT B [redacted] in comparison to the data obtained on the same membranes at the beginning (p=0,03*). No statistical significance has been found between t0 and t1 data of the control group (p=0,20), however the difference between t1 data of product and control group is not significant as well (p=0,33).

Herewith below the graphs of the mean firmness index and mean thickness values over time are reported.

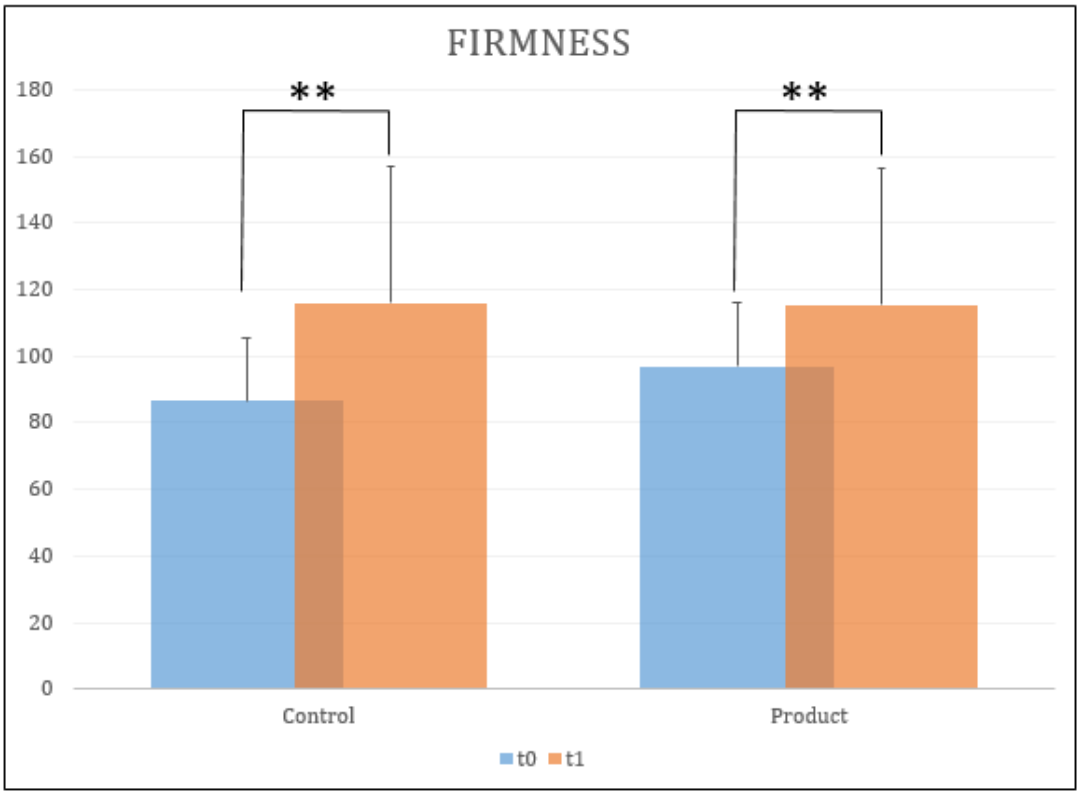

Figure 63. Mean Firmness index at the beginning (t0) and at the end of the study (t1)

The statistical analysis carried out on the samples showed a significant difference in the firmness index of the membranes at t1 in comparison to the data obtained on the same membranes at the beginning (Control group: p=0,003**; Product group: p= 0,008**). However, the difference between t1 data of product and control group is not significant (p=0,48).

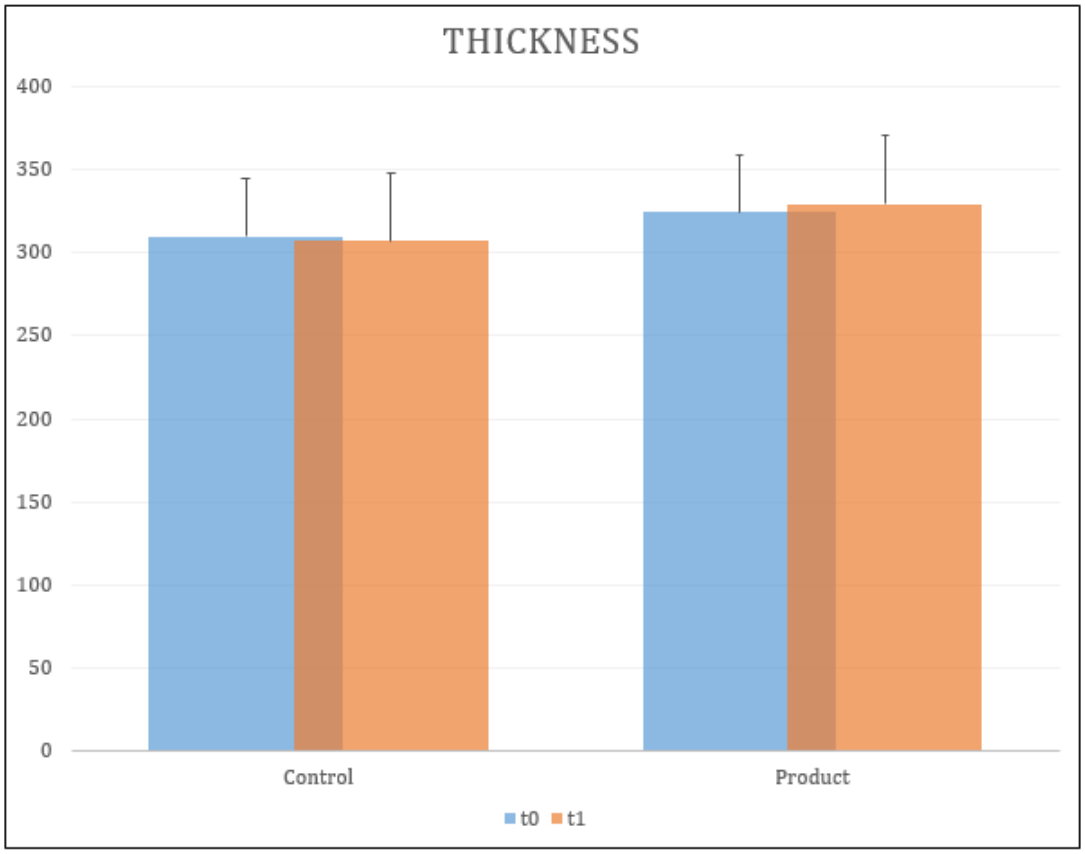

Figure 64. Mean Thickness value at the beginning (t0) and at the end of the study (t1)

The data shows no variation in membranes thickness in both groups.

To summarize, the aim of this work was to evaluate the “in vitro” efficacy of [redacted] PRODUCT B to strengthen and restore the nail plate of soft and flaky nails. In conclusion, after 14 applications of the product on bovine membranes following the protocol described in this report, it is clear that the product induces an improvement in the surface appearance, making it more smooth and homogenous. The variations of the mechanical parameters are summarized in the graph below, which represents the mean of the % variation of the samples for each parameters.

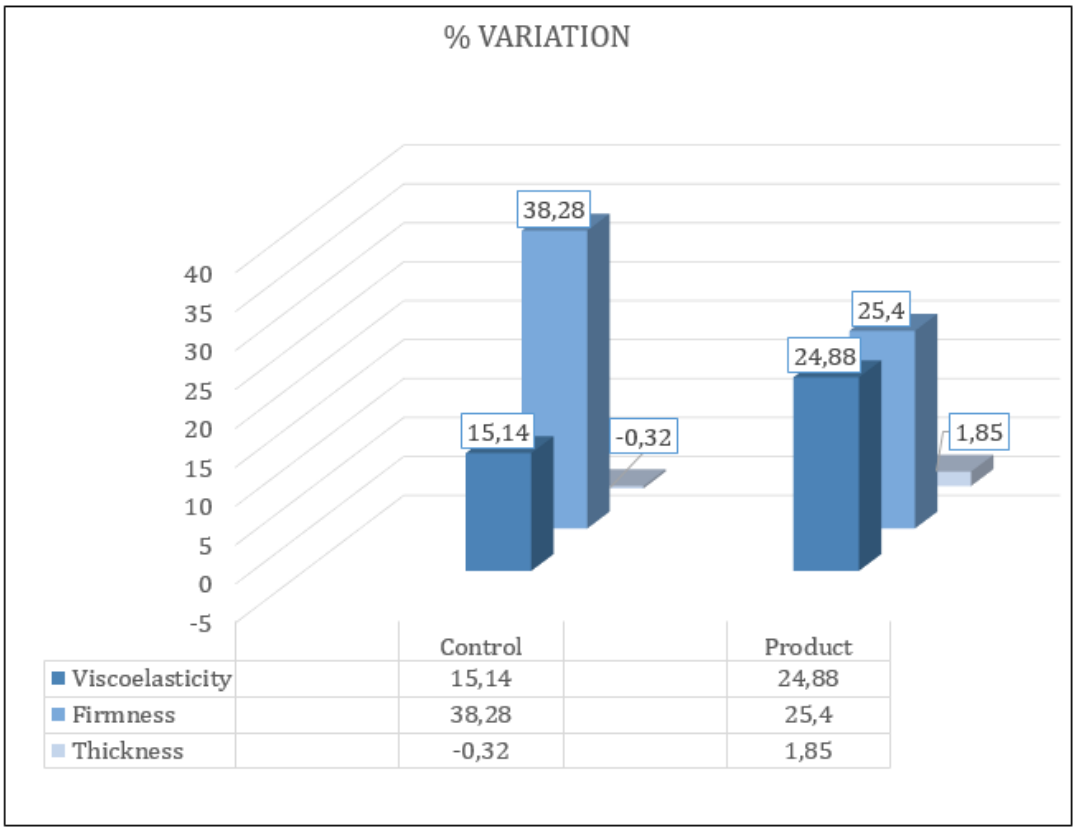

Figure 65. Mean % variation of each parameter over time

In particular, the significant improved viscoelasticity in the product group makes the membranes more shock and crash resilient. The next step would be the application of this protocol to an in vivo study to allow a stronger interpretation of the data obtained.

[redacted] (PRODUCT C)

Materials and Methods

[redacted] PRODUCT C is a product intended to be used on nails that are very dry, too hard and brittle, often due to external drying factors, but also to internal factors. It is described as a well-balanced concentrate based on urea, saccharides and hyaluronic acid, calcium, lemon essential oil and vitamin B5. Its main objectives are to maintain a good moisture balance and to restore and maintain the flexibility of the nail plate. It should also help reinforce structure of nails, which are brittle or altered by drying products or external factors. In order to support the claims we set out an in vitro protocol using membranes obtained from bovine hooves with the suitable mechanical properties required for the product application. For this product in particular, 40 membranes, 20 for each group, with a Firmness Index > 100 were selected and divided in two groups:

- CONTROL group (20 membranes): wash procedure only
- PRODUCT group (20 membranes): [redacted] 478560/998.01

Tables 41-42 report mechanical data obtained from all membranes at the beginning of the experiment. Results are expressed as a mean of at least 3 measurements (SD%: percentage standard deviation; FI: firmness index; VI: viscoelasticity index).

Table 41. Mechanical data obtained from membranes from CONTROL group at the beginning of the experiment (t0). Results are expressed as a mean of at least 3 measurements.

Subject	VI	FI	Thickness
1.13.4	61,71	132,97	303
1.13.7	50,17	139,78	347
1.2.3	33,45	162,65	401
1.8.5	67,87	165,04	315
1.9.9	122,39	256,29	298
2.6.12	76,45	134,39	326
2.7.11	109,50	151,94	306
2.8.12	78,34	233,35	351
3.11.11	112,01	125,12	319
3.11.6	104,91	158,55	338
3.11.9	111,69	147,69	301
3.7.7	73,73	160,46	343
4.10.7	70,81	148,62	320
4.15.5	65,90	117,71	344
4.5.2	60,66	108,93	329
4.7.5	50,07	105,39	348
4.7.7	85,75	179,54	288
4.7.9	124,71	214,74	264
4.8.11	73,38	179,23	321
4.8.3	61,29	175,91	382
Mean	**79,739**	**159,914**	**327,2**
SD%	**32,66**	**24,61**	**9,64**

Table 42. Mechanical data obtained from membranes from PRODUCT group at the beginning of the experiment (t0). Results are expressed as a mean of at least 3 measurements.

Subject	VI	FI	Thickness
1.13.3	101,05	175,8157	267
1.13.6	50,05	169,5086	365
1.13.8	66,77	172,25	314
1.2.4	91,20	131,72	295
1.8.6	87,16	140,29	262
1.8.7	80,35	281,92	306
1.8.8	113,01	201,88	302
2.4.5	62,51	117,61	413
2.7.10	130,28	137,31	304
2.8.11	134,39	164,35	323
2.8.8	60,41	109,39	361
3.11.3	62,52	143,91	354
3.11.7	125,93	140,46	287
3.7.13	63,70	109,32	341
4.10.3	39,98	143,87	363
4.12.5	44,54	161,27	410
4.5.1	105,40	145,31	263
4.7.2	74,05	132,49	374
4.7.6	105,58	157,44	303
4.8.13	54,75	124,04	344
Mean	**82,682**	**153,008**	**327,6**
SD%	**35,45**	**25,07**	**13,74**

[redacted] PRODUCT C was applied to the membranes of the product group every 24 hours; before each application membranes were cleaned with hands-soap, rinsed with warm water following a specific protocol and left drying in a conditioned room. The membranes of the control group were subjected to the cleaning procedure only. After 14 total applications the

membranes were rinsed one last time and then left drying for at least 3 days before the instrumental evaluations (t1).

Results and Discussion

Morphological Evaluation

The membranes where selected with a mixed flaky/smooth surface and they did not show any particular changes after the treatment, both in control and product group. Here in the pictures below there are two examples reported.

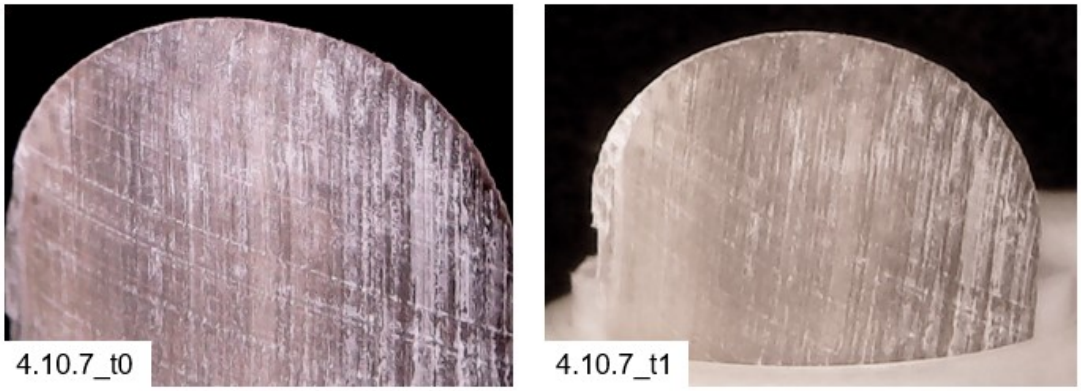

Figure 66. Membrane 4.10.7 (Control Group) before (t0) and after the protocol application (t1).

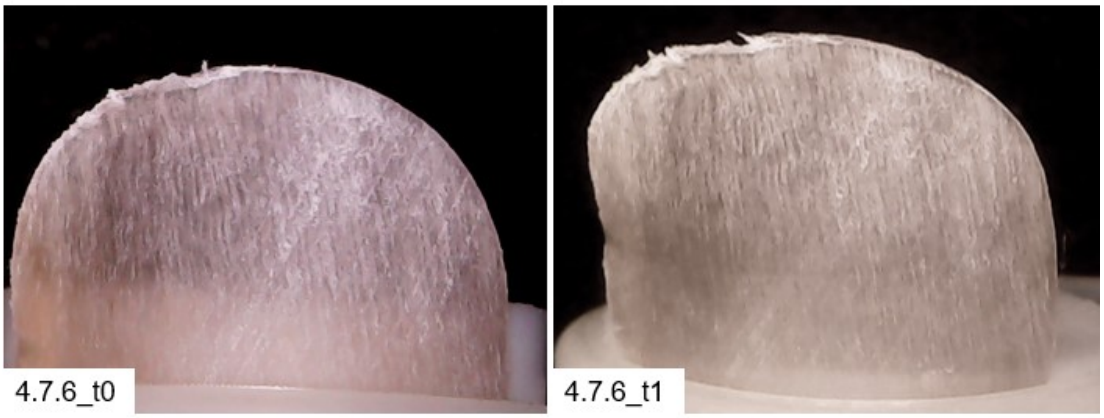

Figure 67. Membrane 4.7.6 (Product Group) before (t0) and after the protocol application (t1).

Mechanical Evaluation

Here in the tables below are reported the values obtained from the instrumental evaluation at the beginning and at the end of the study.

Table 43. Mechanical data obtained from membranes from control group at the beginning (t0) and at the end of the experiment (t1). Results are expressed as a mean of at least 3 measurements.

Membrane N.	*VI_t0*	*VI_t1*	*FI_t0*	*FI_ t1*	*Thickness (µm) t0*	*Thickness (µm) t1*
1.13.4	61,71	95,25	132,97	47,20	303	376
1.13.7	50,17	40,63	139,78	72,14	347	392
1.2.3	33,45	55,34	162,65	89,06	401	392
1.8.5	67,87	50,36	165,04	72,56	315	338
1.9.9	122,39	178,77	256,29	125,39	298	311
2.6.12	76,45	59,41	134,39	80,77	326	352
2.7.11	109,50	129,40	151,94	70,73	306	320
2.8.12	78,34	99,07	233,35	83,82	351	350
3.11.11	112,01	100,97	125,12	98,34	319	343
3.11.6	104,91	95,16	158,55	118,76	338	353
3.11.9	111,69	171,90	147,69	91,63	301	324
3.7.7	73,73	71,15	160,46	104,08	343	368
4.10.7	70,81	82,54	148,62	27,94	320	422
4.15.5	65,90	73,95	117,71	67,99	344	379
4.5.2	60,66	53,27	108,93	52,28	329	397
4.7.5	50,07	130,79	105,39	76,34	348	384
4.7.7	85,74	170,17	179,54	86,19	288	357
4.7.9	124,71	24,66	214,74	48,70	264	455
4.8.11	73,38	153,44	179,23	47,24	321	393
4.8.3	61,29	58,82	175,91	88,24	382	438
Mean	**79,739**	**94,753**	**159,914**	**77,469**	**327,2**	**372,2**
SD%	**32,66**	**49,30**	**24,61**	**31,97**	**9,64**	**10,32**

Table 44. Mechanical data obtained from membranes from product group at the beginning (t0) and at the end of the experiment (t1). Results are expressed as a mean of at least 3 measurements.

Membrane N.	*VI_t0*	*VI_t1*	*FI_t0*	*FI_t1*	*Thickness (µm) t0*	*Thickness (µm) t1*
1.13.3	101,05	185,56	175,82	28,53	267	382
1.13.6	50,05	107,19	169,51	78,63	365	394
1.13.8	66,77	80,29	172,25	28,50	314	419
1.2.4	91,20	116,36	131,72	51,99	295	379
1.8.6	87,16	131,65	140,29	32,68	262	338
1.8.7	80,35	138,10	281,92	126,61	306	318
1.8.8	113,01	162,49	201,88	105,19	302	317
2.4.5	62,52	43,04	117,61	109,98	413	410
2.7.10	130,28	212,53	137,31	49,37	304	393
2.8.11	134,39	157,19	164,35	105,56	323	350
2.8.8	60,41	75,30	109,39	87,68	361	375
3.11.3	62,52	145,60	143,91	78,18	354	390
3.11.7	125,93	173,44	140,46	48,96	287	375
3.7.13	63,70	132,52	109,32	52,51	341	356
4.10.3	39,98	62,66	143,87	56,56	363	421
4.12.5	44,54	58,73	161,27	74,71	410	427
4.5.1	105,40	308,55	145,31	42,57	263	302
4.7.2	74,05	72,46	132,49	90,83	374	395
4.7.6	105,58	165,37	157,44	68,75	303	351
4.8.13	54,75	164,41	124,04	90,24	344	356
Mean	**82,682**	**134,671**	**153,008**	**70,401**	**327,6**	**372,4**
SD%	**35,45**	**46,48**	**25,07**	**41,08**	**13,74**	**9,63**

Herewith below the graph of the mean viscoelasticity index over time for both groups is reported.

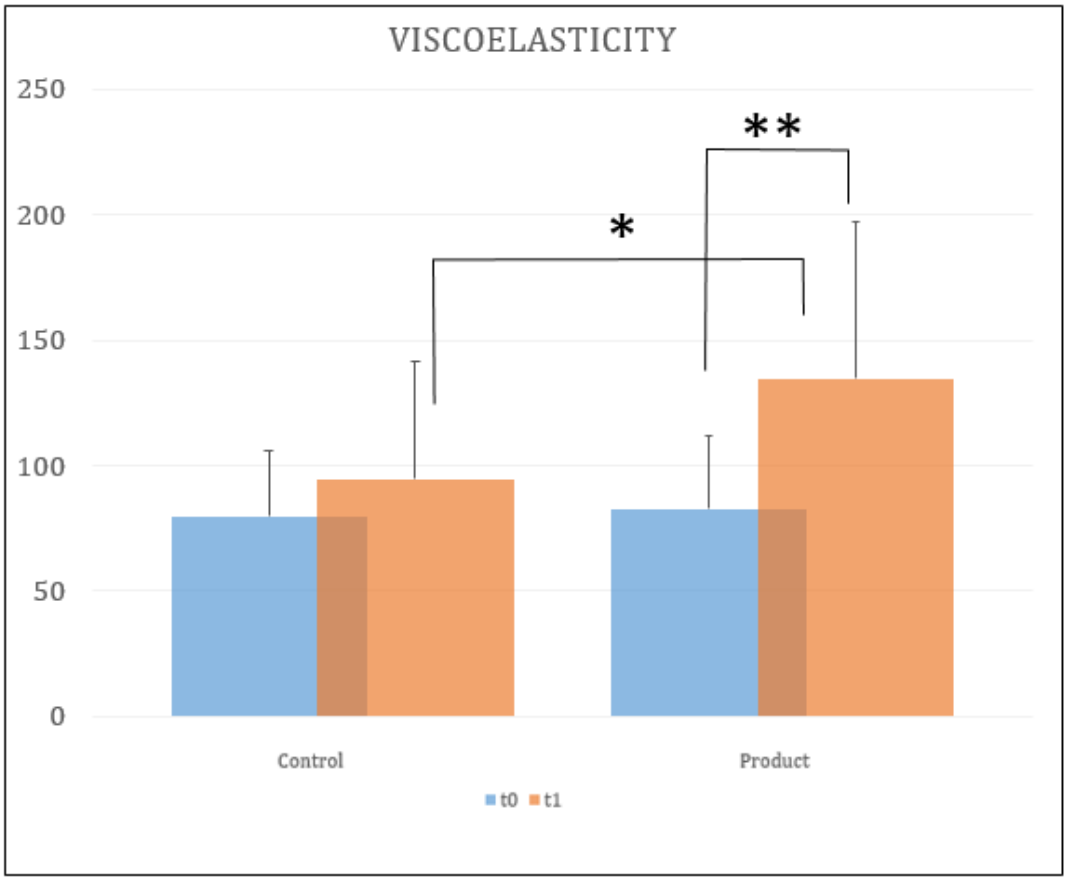

Figure 68. Mean Viscoelasticity index at the beginning (t0) and at the end of the study (t1)

Each value is the mean of all the values that are the result of a mean of at least 3 measurements on each membrane. The statistical analysis carried out on the samples showed a significant difference in the viscoelasticity index of the membranes treated with [redacted] PRODUCT C in comparison to the data obtained on the same membranes at the beginning (p=0,0012**). No statistical significance has been found between t0 and t1 data of the control group (p=0,11), however the difference between t1 data of product and control group is significant as well (p=0,0142*).

Herewith below the graphs of the mean firmness index and mean thickness values over time are reported.

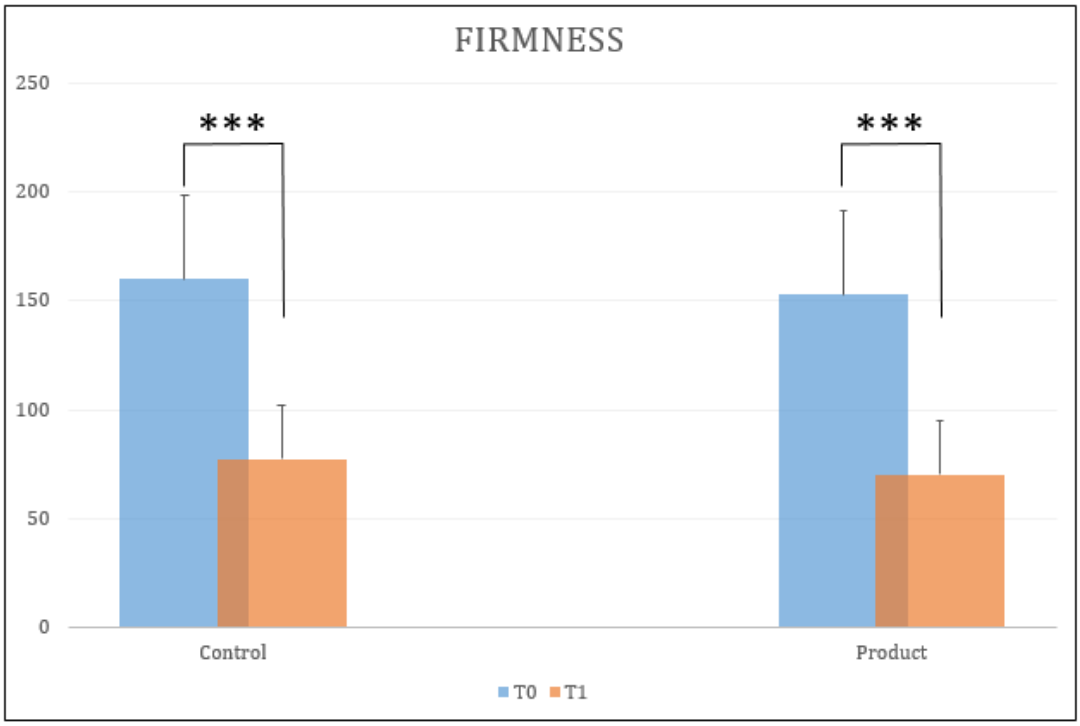

Figure 69. Mean Firmness index at the beginning (t0) and at the end of the study (t1)

Each value is the mean of all the values that are the result of a mean of at least 3 measurements on each membrane. The statistical analysis carried out on the samples showed a strong significant difference in the firmness index of the membranes at t1 in comparison to the data obtained on the same membranes at the beginning (Product group: p= 2,51231E-09***; Control group: p=2,37744E-09***). However, no statistical significance has been found between T1 measurements of product and control group (p=0,21).

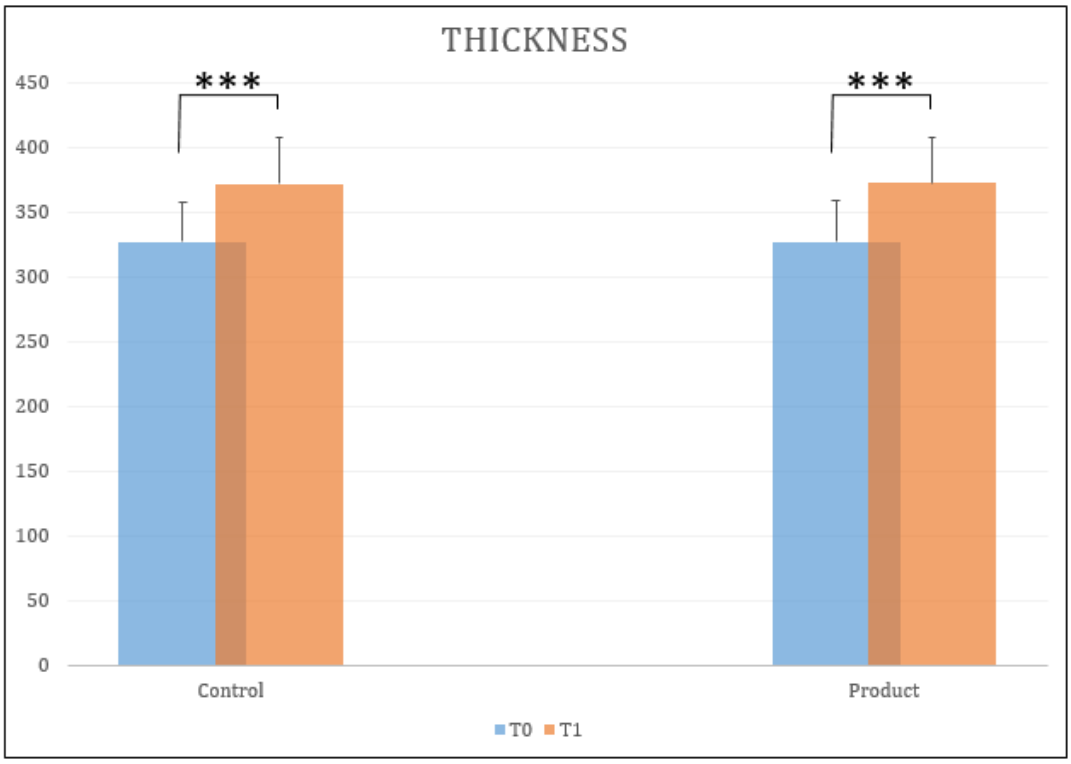

Figure 70. Mean Thickness value at the beginning (t0) and at the end of the study (t1)

Each value is the mean of all the values that are the result of a mean of at least 3 measurements on each membrane. As for the firmness index, the statistical analysis carried out on the samples showed a strong significant difference in the increase of thickness values of the membranes at t1 in comparison to the data obtained on the same membranes at the beginning (Control group: p= 0,00012***; Product group: p= 0,00066***). However, no statistical significance has been found between t1 values of product and control group (p=0,49). The association of the increase in the thickness values and the decrease in the firmness index in both group, with no differences between treated and not treated ones, could be related to the fact that the membranes selected with a high firmness index value, as a model for brittle/dry nails, are very sensitive to water absorption and the cleaning procedure alone induced the hydration of the membranes.

To summarize, the aim of this work was to evaluate the “in vitro” efficacy of [redacted] PRODUCT C to restore and maintain the flexibility of the nail plate.

The variations of the mechanical parameters are summarized in the graph below, which represents the mean of the % variation of the samples for each parameters.

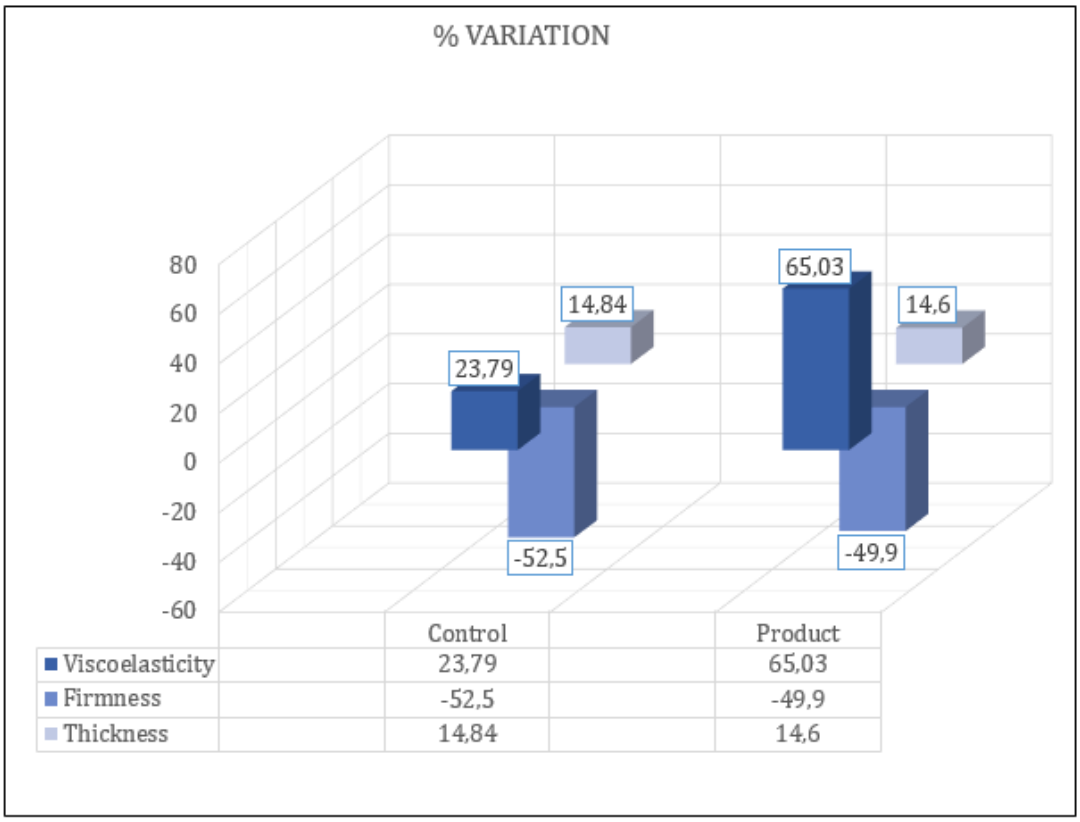

Figure 71. Mean % variation of each parameter over time

After 14 applications of the product on bovine membranes, it is possible to conclude that the product induces a significant increase in the viscoelasticity index of the membranes in comparison to the control group, while the thickness and the firmness increase and decrease respectively with the same percentage in product and control group overtime. To confirm the data, an in vivo study could be suggested.

[redacted] (PRODUCT D)

Material and Methods

[redacted] PRODUCT D is a nutritive cream for damaged nails intended to restore the flexibility to the nail plate and make it more resistant to external shocks; to moisturize the nail plate and to improve the general quality of the nail. In order to support the claims we set out an in vitro protocol using membranes obtained from bovine hooves with the suitable mechanical properties required for the product application. In particular, for this product 36 membranes with a Firmness Index > 100 were selected and divided in two groups:

- CONTROL group (16 membranes): stress/wash procedure only
- PRODUCT group (20 membranes): [redacted] 905.61/049050

Tables 45 report mechanical data obtained from all membranes at the beginning of the experiment. Results are expressed as a mean of at least 3 measurements (SD%: percentage standard deviation; FI: firmness index; VI: viscoelasticity index).

Table 45. Mechanical data obtained from membranes from CONTROL group at the beginning of the experiment (t0). Results are expressed as a mean of at least 3 measurements.

Subject	VI	FI	Thickness
1.12.10	87,14	301,37	347
1.12.4	44,57	175,49	476
1.12.6	80,08	286,80	388
1.12.9	88,52	261,38	374
1.15.3	84,12	247,82	334
1.7.13	36,77	258,52	590
1.7.7	177,15	181,60	259
1.8.12	189,85	241,72	258
1.8.14	84,12	162,20	371
2.10.3	70,47	173,65	391
2.12.10	191,76	230,38	236
2.2.5	79,80	131,90	275
3.7.1	27,59	182,38	544
4.10.4	46,05	215,34	354
4.13.4	75,19	145,22	330
4.15.4	146,23	234,83	213
Mean	**94,337**	**214,412**	**358,8**
SD%	**62,55**	**23,81**	**29,57**

Table 46. Mechanical data obtained from membranes from PRODUCT group at the beginning of the experiment (t0). Results are expressed as a mean of at least 3 measurements.

Subject	VI	FI	Thickness
1.10.3	58,44	207,78	363
1.12.14	79,51	198,41	379
1.12.5	62,89	151,68	431
1.12.8	89,21	166,75	381
1.13.9	75,61	196,10	292
1.7.11	121,25	153,14	298
1.7.3	34,06	199,10	585
1.8.11	186,35	343,91	249
1.8.13	120,48	145,96	262
1.9.11	234,17	180,89	233
2.10.6	112,92	148,08	300
2.12.19	220,25	268,57	260
2.8.4	104,45	135,71	363
3.9.20	75,89	139,94	254
4.10.5	48,30	218,57	406
4.13.8	101,83	162,81	298
4.5.4	115,41	164,58	359
4.7.1	52,35	153,32	477
4.7.12	140,79	275,70	274
4.8.18	210,25	175,06	237
Mean	**112,222**	**189,303**	**335,1**
SD%	**52,58**	**28,18**	**27,18**

[redacted] PRODUCT D was applied to the membranes of the product group for 30 total applications; before each application membranes were strongly cleaned with hands-soap, rinsed with warm water following a specific protocol to mimic not only the standard real-life cleaning of the hands, but also to simulate stress conditions. The membranes were then left drying in a conditioned room. The membranes of the control group were subjected to the cleaning-stress procedure only. After 30 applications, the membranes were rinsed one last time and then left drying for at least 3 days before the final instrumental evaluations (t1).

Results and Discussion

Morphological Evaluation

The membranes where selected with a mixed flaky/smooth surface and at the end of the study the membranes of the product group showed a clear improvement of the surface quality after the treatment (Figure 72), while the membranes in the control group showed no changes or even an increase in the surface deterioration (Figure 73).

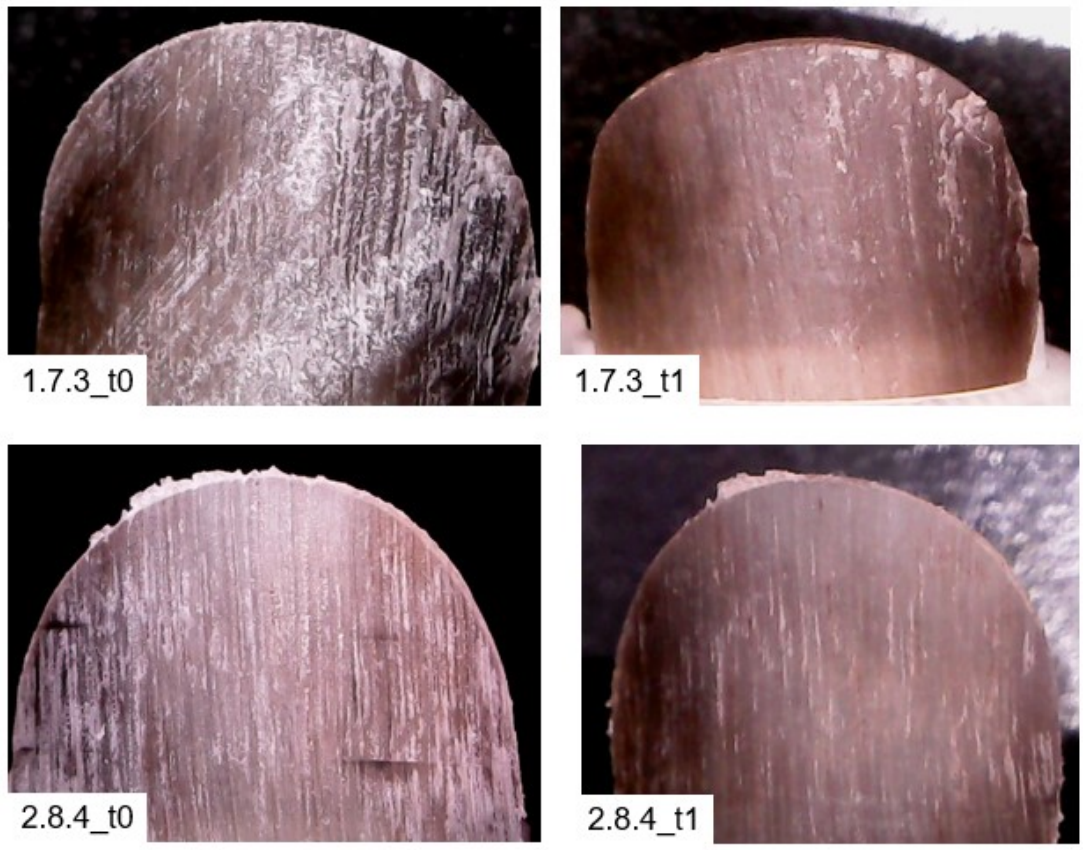

Figure 72. Membranes 1.7.3 and 2.8.4 (Product Group) before (t0) and after (t1) the treatment

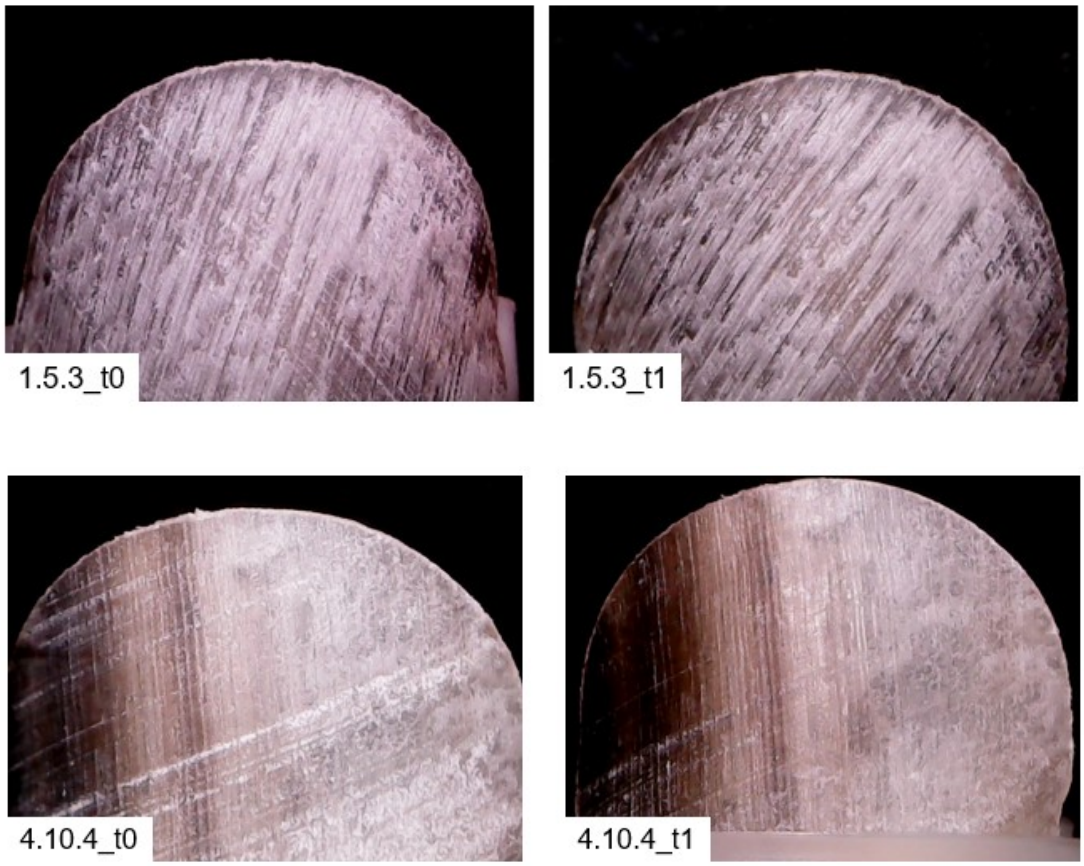

Figure 73. Membranes 1.5.3 and 4.10.4 (Control Group) before (t0) and after (t1) the study

Mechanical Evaluation

Here in the tables below are reported the values obtained from the instrumental evaluation at the beginning and at the end of the study.

Table 47. Mechanical data obtained from membranes from control group at the beginning (t0) and at the end of the experiment (t1). Results are expressed as a mean of at least 3 measurements.

Membrane N.	*Vl_t0*	*Vl_t1*	*Fl_t0*	*Fl_ t1*	*Thickness (µm) t0*	*Thickness (µm) t1*
1.12.10	87,14	87,10	301,37	107,18	347	436
1.12.4	44,57	66,34	175,49	98,80	476	556
1.12.6	80,08	68,31	286,80	144,66	388	445
1.12.9	88,52	74,89	261,38	81,28	374	446
1.15.3	84,12	115,87	247,82	110,69	334	384
1.7.13	36,77	153,86	258,52	87,60	590	472
1.7.7	177,15	156,99	181,60	62,06	259	359
1.8.12	189,85	39,92	241,72	117,40	258	319
1.8.14	84,12	69,35	162,20	75,82	371	502
2.10.3	70,47	96,20	173,65	61,09	391	520
2.12.10	191,76	164,16	230,38	65,45	236	351
2.2.5	79,80	76,35	131,90	105,96	275	348
3.7.1	27,59	82,82	182,38	48,22	544	674
4.10.4	46,05	30,96	215,34	34,30	354	485
4.13.4	75,19	198,26	145,22	46,71	330	406
4.15.4	146,23	128,60	234,83	65,39	213	286
Mean	**94,337**	**100,624**	**214,412**	**82,038**	**358,8**	**436,8**
SD%	**62,55**	**52,62**	**23,81**	**36,72**	**29,57**	**22,66**

Table 48. Mechanical data obtained from membranes from product group at the beginning (t0) and at the end of the experiment (t1). Results are expressed as a mean of at least 3 measurements.

Membrane N.	*VI_t0*	*VI_t1*	*FI_t0*	*FI_t1*	*Thickness (µm) t0*	*Thickness (µm) t1*
1.10.3	58,44	102,24	207,78	146,04	363	425
1.12.14	79,51	124,38	198,41	168,91	379	435
1.12.5	62,89	77,51	151,68	157,79	431	466
1.12.8	89,21	63,39	166,75	220,48	381	390
1.13.9	75,61	77,48	196,10	282,71	292	342
1.7.11	121,25	46,69	153,14	114,27	298	351
1.7.3	34,06	56,06	199,10	150,22	585	659
1.8.11	186,35	202,01	343,91	165,35	249	337
1.8.13	120,48	127,14	145,96	108,34	262	374
1.9.11	234,17	49,36	180,89	117,15	233	308
2.10.6	112,92	68,89	148,08	75,18	300	400
2.12.19	220,25	219,55	268,57	203,73	260	343
2.8.4	104,45	40,36	135,71	154,24	363	400
3.9.20	75,89	158,37	139,94	53,37	254	437
4.10.5	48,30	65,11	218,57	96,14	406	398
4.13.8	101,83	54,42	162,81	59,58	298	380
4.5.4	115,41	97,50	164,58	123,84	359	408
4.7.1	52,35	130,45	153,32	91,11	477	520
4.7.12	140,79	136,69	275,70	136,22	274	361
4.8.18	210,25	34,77	175,06	44,80	237	430
Mean	**112,222**	**96,619**	**189,303**	**133,475**	**335,1**	**408,2**
SD%	**52,58**	**54,80**	**28,18**	**44,23**	**27,18**	**18,88**

Herewith below the graph of the mean viscoelasticity index over time for both groups is reported.

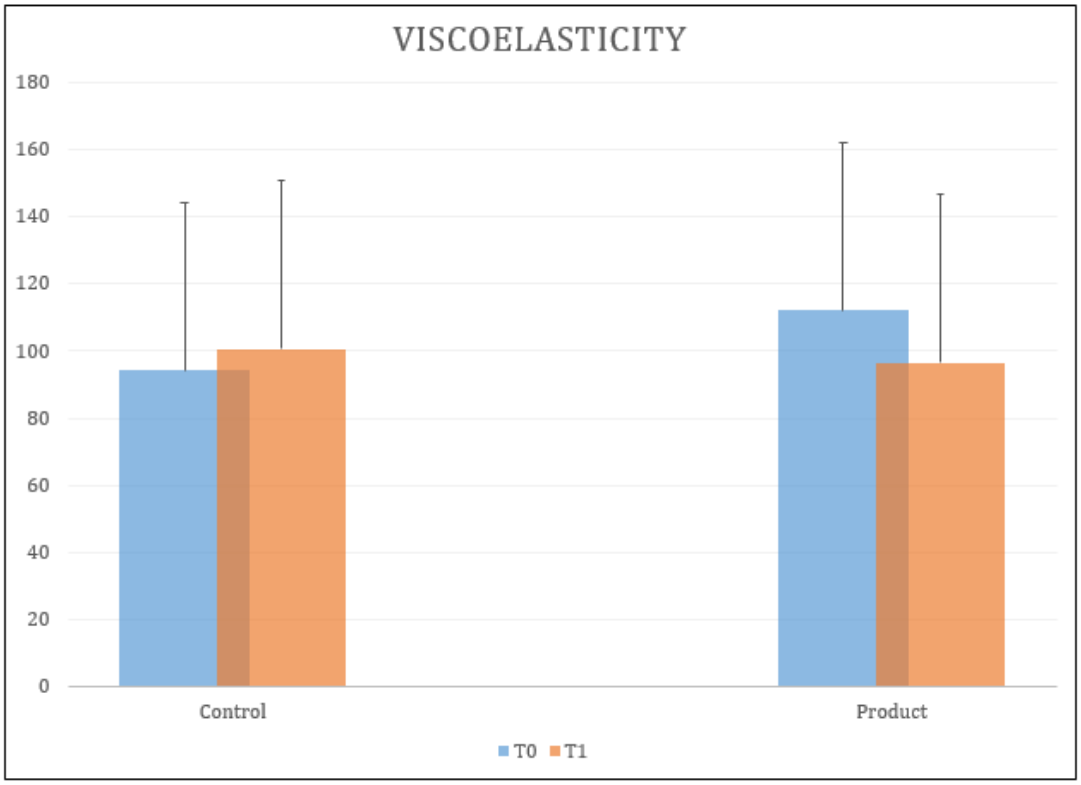

Figure 74. Mean Viscoelasticity Index at the beginning (t0) and at the end of the study (t1)

Each value is the mean of all the values that are the result of a mean of at least 3 measurements on each membrane. The results shows no changes after the treatment.

Herewith below the graphs of the mean firmness index and mean thickness values over time are reported.

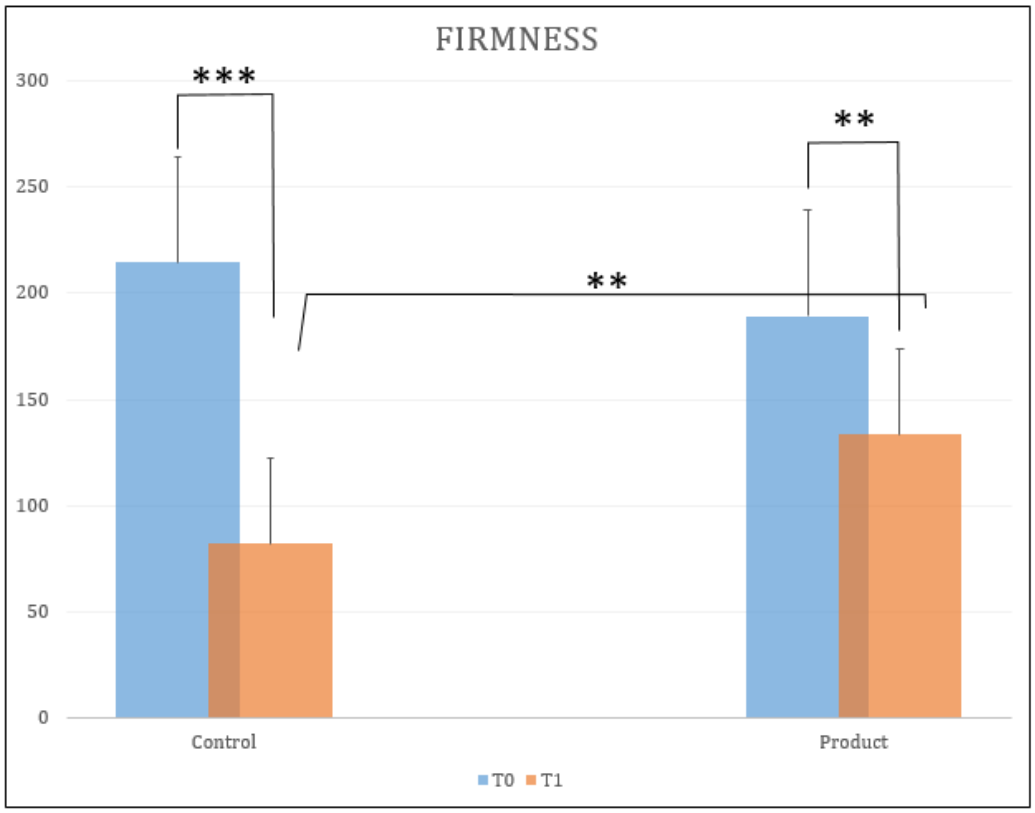

Figure 75. Mean Firmness Index at the beginning (t0) and at the end of the study (t1)

Each value is the mean of all the values that are the result of a mean of at least 3 measurements on each membrane. The statistical analysis carried out on the samples showed a significant difference in the firmness index of the membranes at t1 in comparison to the data obtained on the same membranes at the beginning (Control group: p=2,12061E-09***; Product group: p=0,0016**) which decreases over time. Moreover, the difference between t1 measurements of product and control group is significant as well (p=0,001**). The strong reduction in the firmness index of the control group could be a direct consequence of the stress induced on the membranes by the strong cleaning procedure applied; the product application seems to have led to a reduction of the index as well, which is positive since the properties of the samples selected at the beginning (brittle/dry nails). Moreover, the product seems to exert also protective effects on the membranes, leading to a decrease in the firmness index but no to a value that could be too low, making the membranes too soft, which is what is starting to happen in the control group.

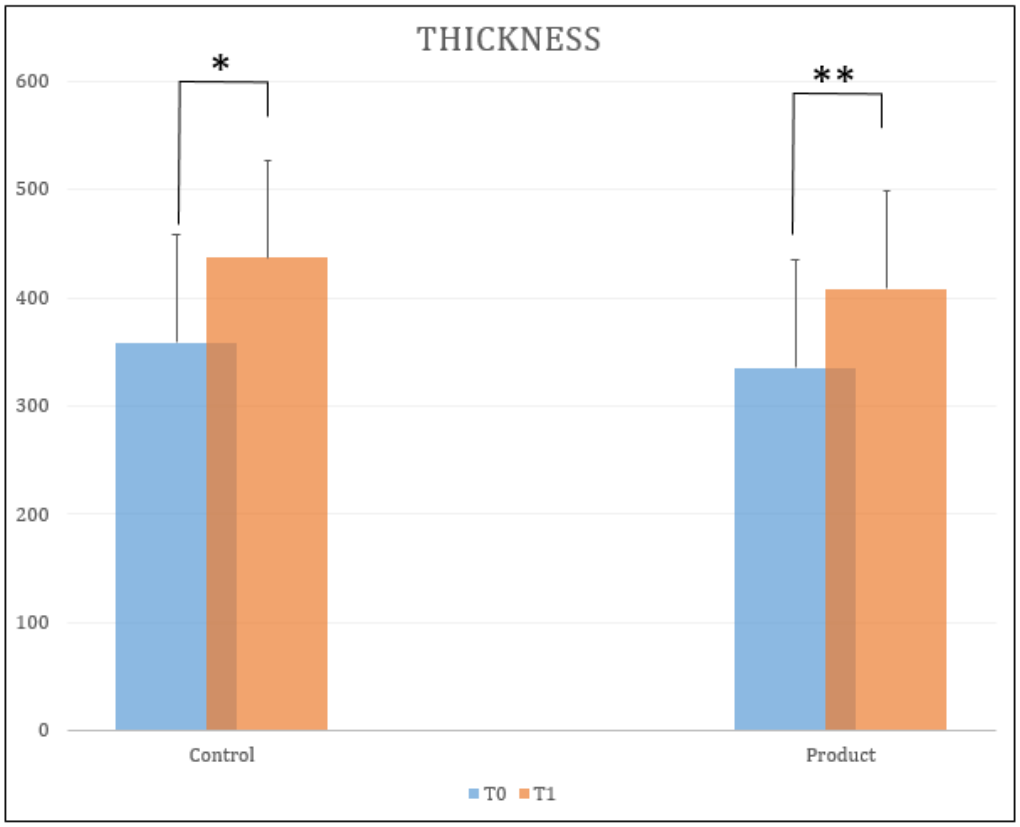

Figure 76. Mean Thickness value at the beginning (t0) and at the end of the study (t1)

Each value is the mean of all the values that are the result of a mean of at least 3 measurements on each membrane. The statistical analysis carried out on the samples showed a significant difference in the increase of thickness values of the membranes at t1 in comparison to the data obtained on the same membranes at the beginning (Control group: p= 0,02*; Product group: p= 0,005**), probably due to a standard hydration effect on the membranes. However, no statistical significance has been found between t1 values of product and control group (p=0,18).

To summarize, the aim of this work was to evaluate the “in vitro” efficacy of [redacted] PRODUCT D.

After 30 applications of the product on bovine membranes, it is possible to conclude that the product exerts a protective effect, since it induces a significant decrease in the firmness index of the membranes which at the beginning were too hard. However, the index did not drop too low, making the membranes too porous, as it happens in the control group. Moreover, the

product induced an improvement in the surface appearance which appears more smooth and homogenous. This interpretation of the results could be confirmed through an in vivo study.

COLOUR ASSAY

Material and Methods

The aim of this study was to evaluate in vitro and in vivo efficacy of two [redacted] base coats to prevent yellowing and pigmentation of nails after use of coloured nail polish.

Tested products:

- [redacted] BASE 1 (904.03, 359231)
- Mavala 002® (902.03, 409073)
- Mavala Roma 187® as reference nail polish

[redacted] BASE 1 claims to prevent dehydration, splitting, flaking and chipping of nails and to help them to retain their flexibility. [redacted] BASE 1 has a water-based formula which forms a protective barrier between the nail plate and nail coloured polish. In this way it should prevent yellowing of nails and improve adherence of nail polish.

The second product, MAVALA 002®, has a solvent based formula which claim to isolate the nail plate, in order to prevent pigments in nail polish from coming in direct contact with the nail, protecting it against yellowing. On the other hand, its slightly "sticky" formula allows perfect adhesion of nail polish and extends manicure duration.

Colorimeter Assay

The first step was to measure colour index in vitro to establish a correlation between the colour of the nail polish used for the study and the colour after application on the samples (both in vitro and in vivo).

The Colorimeter CL 400 measures specifically the colour of the skin, but it can also be used on skin appendix. Measuring values are expressed as coordinates in the colour space L*a*b* or as RGB (red/green/blue).

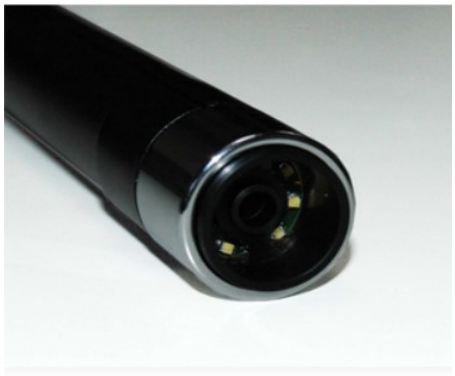

Figure 77. Colorimeter CL 400 probe

In order to do this, a thick and uniform layer of the polish has been lied on a petri dish graphically divided in three areas:

1. [redacted] COLOUR alone
2. [redacted] BASE 1 + [redacted] COLOUR
3. Mavala 002 + Roma 187

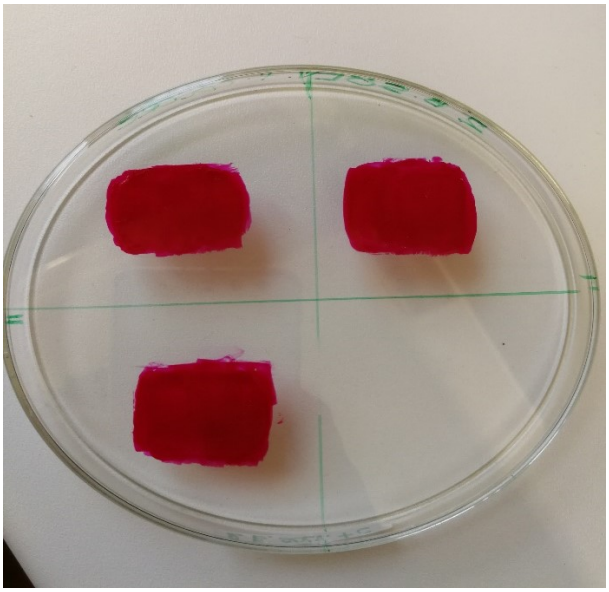

Figure 78. Petri dish areas with mail polish applied

For each area the colour indexes have been measured in triplicate with the colorimeter, and for each index we obtained a mean value.

Table 49. Mean values of colour index for each area

	R	G	B
Colour alone	127,78	68,33	80,22
Base 2 + colour	126,89	72,78	81,11
Base 1 + colour	127,22	75,78	83,39

In order to have a reliable correlation, the percentage variation between this measurements and the ones of the colour applied on the sample (in vitro and in vivo) must not exceed 10%.

The variations have been calculated using this equation:

$V\% = [(Vf – Vi) / Vi]*100$

Vf = index mean of the colour applied on the sample (in vitro/in vivo)
Vi = index mean of the colour on the petri dish

In Vitro Study

Membranes selection

For this study 28 membranes of about 300 µm thickness have been selected, all with smooth surface.

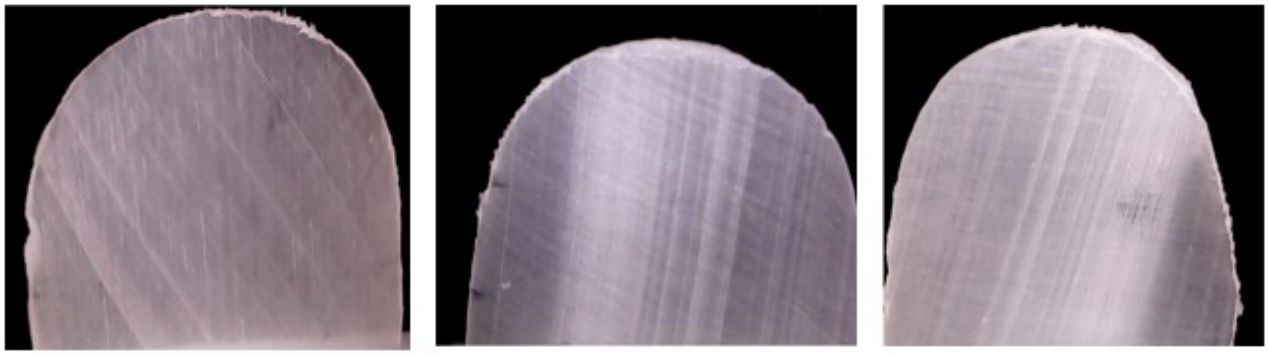

Figure 79. Smooth membranes selected for the study

They have been classified in three groups according to the wettability properties of the surface based on the percentage variation of the angle over time (from 0 to 60 seconds):

- VERY WETTABLE: 80-100% reduction (12 membranes total)
- WETTABLE: 40-79% reduction (11 membranes total)
- POORLY WETTABLE: 0-39% reduction (5 membranes total)

In the table below are reported the mean angle values at the different time points expressed in degrees and their standard deviation.

Table 50. Mean angle values for each group of membranes

	VERY WETTABLE		WETTABLE		POORLY WETTABLE	
Time	Mean	SD	Mean	SD	Mean	SD
0s	70,97	6,92	83,85	13,42	67,4	13,68
30s	9,5	9,68	42,87	13,59	53,95	6,94
60s	2,29	3,01	29,92	11,16	48,8	13,13

Some of the membranes were divided in three treatment groups (3 membranes each group), as for we did with the petri dish, values of which have been used for correlation check.

- Group 1: Application of coloured nail polish [redacted] COLOUR only;

- Group 2: Application of BASE 2 + COLOUR;
- Group 3: Application of BASE 1 + COLOUR.

The colorimeter analysis has been performed on the natural membranes, on the membranes with products on and after product removal. All the products have been removed with the same acetone based polish remover.
The mean colorimetric index for the natural membranes are reported in the table below.

Table 51. Colorimetric indexes mean values for natural membranes

	R	G	B
Mean	152,00	151,00	151,22
SD	1,41	1,50	0,83

In Vivo Study

The same protocol applied on the membranes for the in vitro study has been used in vivo on 9 volunteers. All the participants were healthy subjects aged 22-30 years old, with no detectable nail problems and no products applied on the nail for at least 3 days.
As for the in vitro study, the colorimeter measurements have been taken on the natural nail, on the nail after the application of the products and after the removal. All the products have been removed with the same acetone based polish remover. All the measurements are performed in triplicates.
To simplify results explanation also in this case the measures are divided in three groups:

- Group 1: Application of coloured nail polish [redacted] only;
- Group 2: Application of BASE 2 + COLOUR;
- Group 3: Application of BASE 1 + COLOUR.

Every volunteer took part in each group.

Results and Discussion

In Vitro Study

The percentage variation of the colour on the membranes in all three group has a good correlation with the index measured for the petri dish. Here in the table below are reported the values of the calculated variations and their mean and they are within the 10% limits.

Table 52. Colorimetric indexes mean values after application of the products

	R	G	B
Group 1	130,22	80,00	90,33
Group 2	131,44	80,83	87,89
Group 3	132,33	82,11	89,55
Mean	131,33	80,98	89,26
SD	2,56	3,51	3,25

Table 53. Percentage variation of index values of membranes colour in comparison to petri dish colour

	R	G	B
Group 1	11%	8,30%	5,40%
Group 2	7%	5,20%	0,80%
Group 3	5,20%	0,60%	1,50%
Mean	**8%**	**5%**	**3%**

The products have been left applied on the membranes for 4-6 hours.
After removal of the nail polish from the membranes the appearance is clearly different: group 1 membranes where the polish has been applied with no base coat under have more pigment left on in comparison to membranes of group 2 and group 3 (Figure 80).

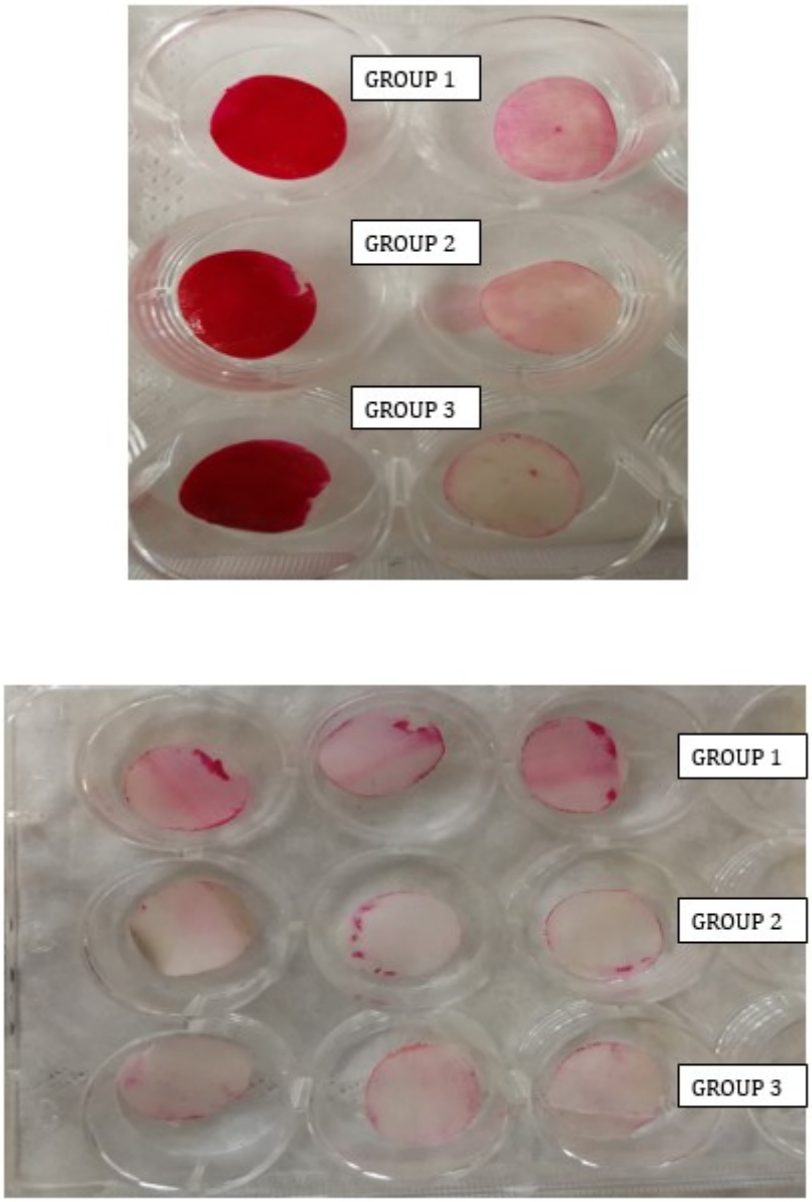

Figure 80. Membranes of each group before and after removal of the products

The colorimeter analysis objectively confirm the results, since the percentage variation of the index values after the removal of the products in comparison to the natural membranes index are higher for Group 1 then Group 2 and Group 3.

Table 54. Colorimetric indexes mean and standard deviation values for each group after product removal

	R	G	B
Group 1	141,89	116,89	127,00
Group 2	156,22	143,44	146,75
Group 3	148,89	136,55	142,77

Table 55. Mean percentage variation of RGB indexes for each group after products removal in comparison to the original values

	R	G	B
Group 1	6,65%	22,59%	16,01%
Group 2	5,85%	5,00%	3,88%
Group 3	2,05%	9,57%	5,58%

In Vivo Study

In the tables below are reported the mean values of the colorimetric indexes RGB with the products applied to compare to the petri dish results as reference.

Table 56. Colorimetric indexes mean values after application of the products

	R	G	B
Group 1	147,80	68,61	87,07
Group 2	143,26	90,33	95,04
Group 3	144,19	68,70	86,00
Mean	**145,08**	**75,88**	**89,37**
SD	**2,40**	**12,52**	**4,94**

Table 57. Percentage variation of index values of membranes colour in comparison to petri dish colour

	R	G	B
Group 1	29,30%	7,98%	14,40%
Group 2	20,72%	17,65%	16,21%
Group 3	19,26%	13,40%	4,74%
Mean	**23,09%**	**13,01%**	**11,78%**

In this case we have higher percentage variation of the indexes in comparison to the petri dish data, which is probably due the contribution of the colour of the skin underneath the nails.

The products have been left on by volunteers for 4-6 hours.

After removal of the nail polish the colorimetric indexes have been measured again and they have been compared to RGB index of the natural nails.

Table 58. Mean colorimetric index and standard deviation values after products application

		R	G	B
Group 1	**Mean**	180,48	137,00	140,67
	SD	5,08	7,59	4,90
Group 2	**Mean**	177,593	136,56	134,48
	SD	5,72	6,86	17,57
Group 3	**Mean**	177,78	137,85	133,78
	SD	5,86	5,15	15,83

Table 59. Mean percentage variation of RGB indexes for each group after products removal in comparison to natural nails values

	R	G	B
Group 1	3,29%	5,91%	4,64%
Group 2	1,86%	3,68%	3,73%
Group 3	1,72%	2,45%	1,97%

Also in this case the variation is lower for group 2 and 3 then in group 1.

To summarize, the aim of this work was to evaluate the performances of two base coat formulations, [redacted] BASE 2 and [redacted] BASE 1, to support their commercial claims through in vitro and in vivo studies. The results obtained were positive, in particular with in vitro protocol. However, to repeat the experiment with a higher number of samples will allow a statistical analysis of the results. The in vivo study results are promising as well: to have more solid evidence of the efficacy in vivo it could be useful to have a longer application time and repeated application.

CONCLUSION

In this chapter, the results of three different kinds of assessment applied to several products have been proposed. In particular:

- mechanical properties assessments applied to both “soft nail” in vitro model (products tested: [redacted] (PRODUCT A) and [redacted] PRODUCT B) and “hard nail” in vitro model (products tested: [redacted] PRODUCT C and [redacted] PRODUCT D);
- permeability assessment (product tested: [redacted] PRODUCT A);
- colorimeter assay (products tested: [redacted] BASE 1 and BASE 2 [redacted]).

It can be stated that these in vitro studies objectively sustained most of the commercial claims of those products and in general bovine hooves membranes demonstrated their suitability as in vitro model to assess efficacy in this field.

Permeability and colorimeter assays protocols were both effective, giving promising results. Repeating the experiments with a higher number of samples would allow a stronger data analysis.

Mechanical properties assessments on nails are tricky and complicated, since it is quite hard to find references in literature about previous works in the field, in particular for in vitro assessment. Moreover, human nails and nail models are difficult samples to work with: apart from the fact that working with biological derivatives involves a higher natural variability in the data, nails and other similar are extremely sensitive to external factors.

A “Soft nail” model has been chosen for testing of [redacted] PRODUCT A and [redacted] PRODUCT B. [redacted] (PRODUCT A), a solvent-based nail polish, application induced an improvement in the surface appearance of the membranes, making it more smooth and homogenous and a significant increase in the firmness index of products groups in comparison to the placebo group. [redacted] PRODUCT B, a water base product, as a main effect induced an improvement in the surface appearance, making it more smooth

and homogenous. There were some differences in the two protocols applied: concerning mechanical properties in the first case the membranes suitable for the study were selected based on their VI (>100) while for the second product they have been selected based on both FI (<130) and VI (>74). For both studies membranes were selected with flaky surface. The removal procedure was also different since for solvent-based nail polish an acetone-based solution was needed to remove the product. Moreover, for the first product were used formulations with and without active ingredients to be compared to each other, while for [redacted] PRODUCT B experiment the effect of the formulation was compared to the wash procedure alone.

“Hard nail” model was chosen to test [redacted] PRODUCT C and [redacted] PRODUCT D efficacy, both presented as emulsion, through the selection of membranes with FI >100 and a mixed of flaky and smooth surface. As main result, [redacted] PRODUCT C which induced a significant increase in the viscoelasticity index of the membranes in comparison to the control group and [redacted] PRODUCT D induced an improvement in the surface appearance of membranes and a significant decrease in the firmness index. In both experiments product efficacy was compared to the washing procedure as a control, which was stronger and longer for [redacted] PRODUCT D to mimic a stress condition. The association of the increase in the thickness values and the decrease in the firmness index have been observed in both group. As explained before, this could be related to the fact that the membranes selected with a high firmness index value, asa model for brittle/dry nails, seem to be very sensitive to water absorption and the cleaning procedure alone induced the hydration of the membranes. The [redacted] PRODUCT D study was longer, exacerbating this result, but also pointing out the potential protective action of the product, which lead to a lower decrease of the firmness index in comparison to the control group.

In general, the intrinsic variability of the model properties has been controlled in a satisfactory way with strict selection criteria for the suitable membranes to be used for the studies. This could suggest that the difficulties faced in these experiments could be overcome adding more selection criteria to be satisfy, such as a deeper analysis of surface properties of the membranes, such as contact angle values to assess compatibility with the formulation to be applied,

like it has been done for permeability assay. Water content and TOWL could be other two parameters to be considered both when selecting membranes at the beginning and as an endpoint of the study. It is crucial also to take into account the time required by the membranes production and selection processes which would be elongated by these additions, so it would require a different organization of the samples groups.

Moreover, the general results obtained with these in vitro efficacy assays suggested that the use of a placebo formulation, when available, allows a more reliable comparison among groups, removing the bias due to the action of the formulation excipients.

CHAPTER 3

PHARMACEUTICAL APPLICATIONS

INTRODUCTION

Fungi constitute a very heterogeneous group of organisms and from the microbiological point of view two morphological kind are important, moulds and yeasts. In nature, saprophytic fungi are predominant, however, in some cases, they may parasitize living organisms and cause infectious diseases. The most common are onychomycoses which are more frequent in people with other skin and nail disorders and in immunocompromised subjects. Fungal infections can be caused by dermatophytes such as *Trichophyton rubrum* or some types of yeasts as *Candida albicans*. Despite the high frequency of these infections within the population, the treatments option remain poor, and given the peculiarity of the affected area, new products are challenging to be assessed.

In this chapter, three different studies are reported. Each of them address a different step in the efficacy assessment of a product designed to be used for onychomycoses prevention or treatment, in an area where the assessment protocols are scares and not yet standardized.

The first study is a in vitro efficacy assessment of drugs alone and in combination against Trichophyton rubrum, aiming to find the most promising combination and to compare two different methodologies.

The second study is a permeability in vitro study of a product through the use of an innovative apparatus, aiming to assess both the feasibility of the novel method and the effects of the vehicles on the permeability rate of the active.

The last study has the aim to develop an innovative protocol to assess anti-adhesive effect within fungal nail infections.

EFFICACY OF DRUGS ALONE AND IN COMBINATION AGAINST T.RUBRUM

Dermatophytes are one of the most common causing agents of onychomycoses, which is a wide diffuse disease often challenging to be successfully treated. A novel approach would be to use a carrier to convey a combination therapy to the target. In order to develop it, the first step is to assess the efficacy of several drug combinations against fungal cell growth. However, there is a lack of standardized antifungal susceptibility test for dermatophytes. In this study, the aim is to assess efficacy of three drugs (Terbinafine, Amorolfine and Ciclopirox) alone and in combination against *Trichophyton Rubrum* using two different techniques: broth microdilution test and Disc Diffusion assay.

Materials and Methods

Materials

Trichophyton Rubrum strain ATCC 28188 was obtained from Thermo Scientific. The three antifungals used for the study were: amorolfine HCl (Batch KL/AMR-7/001/04-D) purchased from Ranbaxy Research Laboratories, India; ciclopirox olamine (2012040) purchased from Insight biotechnology Ltd, UK and terbinafine HCl 98% (78628-80-5) purchased from AK scientific, USA. Sabouraud Dextrose Broth (BCBR4280V), RPMI -1640 Medium (SLBT7944) and MOPS ≥ 99.5% titration (BCBT6515) were purchased from Sigma Aldrich, UK. Dimethyl sulfoxide (DMSO) (1491285) and Methanol 99.9% (199381) was purchased from Fisher, UK.

Preparation of media

Sabouraud Dextrose Agar (SDA) was used to prepare agar plates for the growth of T. Rubrum. Using an analytical balance, 26g of SDA powder were accurately weighed and placed into a 500mL Duran bottle with 400mL distilled water. The bottle was tightly sealed and shaken well for even suspension. After autoclaving the solution at 121°C for 2 hours, the solution was cooled down and poured into sterilized petri dishes in a microbiological safety cabinet to maintain sterility. Once the petri dishes had solidified at room temperature they were stored at 4°C ready to be used.

Using an analytical balance, 10.4g of RPMI-1640 was weighed and dissolved in 900mL of distilled water under constant stirring. Then 34.53g of MOPS buffer were add to the solution. Once the powders achieved complete dissolution, the pH was measured using a Thermo Scientific pH meter. Maintaining the solution under constant stirring, sodium hydroxide 1M was added into the solution to achieve a pH of 7. Once achieved the desired pH value, additional distilled water was added to have a final volume of 1L of RPMI -1640 medium. The solution was autoclaved at 121°C for 2 hours and stored in the fridge at 4°C after cooled down at room temperature.

Preparation of inoculum

T. rubrum (Strain ATCC28188) isolate was used in this study. Using sterile inoculating loops, a swab of T. Rubrum was taken and streaked onto the prepared SDA agar plates and incubated for 7 days at 32°C.

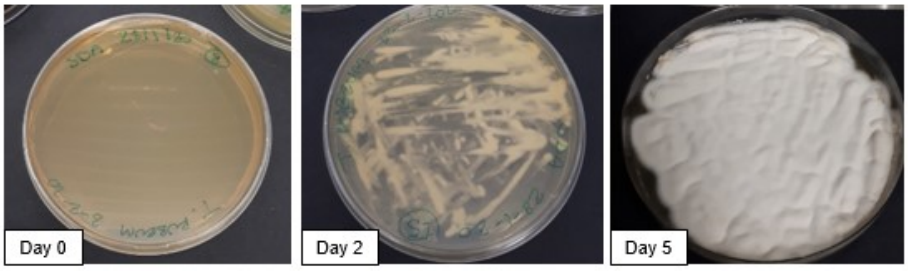

Figure 81. Different stadium of growth of T. Rubrum

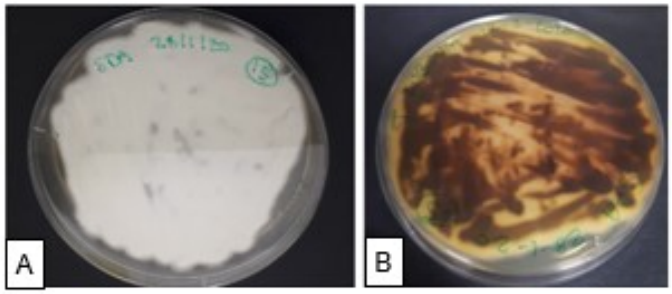

Figure 82.Pictures of fully grown T. Rubrum from the top (A) of the plate and from the bottom (B)

The inoculum for the efficacy assays has been prepared right before the begin of the experiment using the following procedure: around 5-10ml of sterile saline solution (0,85% NaCl in distilled water) was pipetted on to the fungal colonies on the plate. The surface of the agar was then scrapped under saline solution

using a sterile loop to form a suspension of dermatophyte hyphae and conidial. A disposable 5ml plastic syringe was then used to draw the fungal suspension and to filter is through an autoclaved stainless steel filter with Whatman's filter paper 40 (diameter 110mm) attached. This was made in order to remove all dermatophyte hyphae and ensure that only micro-conidia were left in the suspension. The suspension was vortexed for 15 seconds before turbidity checks were carried out by using a UV spectrometer. The instrument blank was made using saline alone.

The target absorbance was 0.15-0.17au, a similar value that was given by McFarlands solution. More saline or more suspension was added until the solution meets the target reading. The final concentration obtained was from 0.4×10^4 to 5×10^4 colony forming units (CFU)/ml.

This solution was used without further dilution to perform disc diffusion tests. For broth microdilution assay the fungus stock solution was further diluted by 50 folds with RPMI-1640 medium.

Preparation of drugs

Stock solutions of terbinafine HCl, amorolfine HCl and ciclopirox olamine were prepared using methanol, as the drugs are insoluble in water. Methanol is an ideal solvent in particular for disc diffusion assay, as it is a volatile solvent and will evaporate, leaving the antifungal drug only on the disc, to diffuse on the agar. The stock solutions were then diluted at least 1:100 with RPMI for Broth Microdilution experiment. This was to ensure that the final concentration of solvents in the well does not exceeds 1% w/v.

Disc diffusion susceptibility test

This method is based on solid agar plates to determine the zone of inhibition (ZOI) of antifungal drugs.

Single drug testing

Prior to the experiment, all discs required for disc diffusion experiment were placed in a small Duran bottle and autoclaved at 121°C for 2 hours. During the experiment, discs were placed on a sterile empty petri dish and were prepared in a safety cabinet.

Each petri dish was divided in quadrants, 3 spaces were used for the drug and 1 for the control. Based on previous experiments two different protocols were used:

Option A: 20µL of prepared drug stock solution was pipetted on each disc, using 20µL of pure methanol as a control;

Option B: 10µL of prepared stock solution + 10µL of methanol was pipetted onto each discs using a Gilson pipette to achieve a total volume of 20µL of volume per disc. 20µL of methanol was used as a solvent control.

Whilst the discs were left to dry allow methanol to evaporate from the disc, 100µL of fungal inoculum was loaded onto the SDA petri dish. The inoculum was spread by a sterile disposable spreader to evenly distribute the inoculum on the petri dish. Once the prepared discs were thoroughly dry, sterile tweezers were used to transfer the discs onto the petri dish. The petri dish was then incubated at 32°C for 7 days. One plate without discs has been prepared for each experiment as a spreading/fungal growth test.

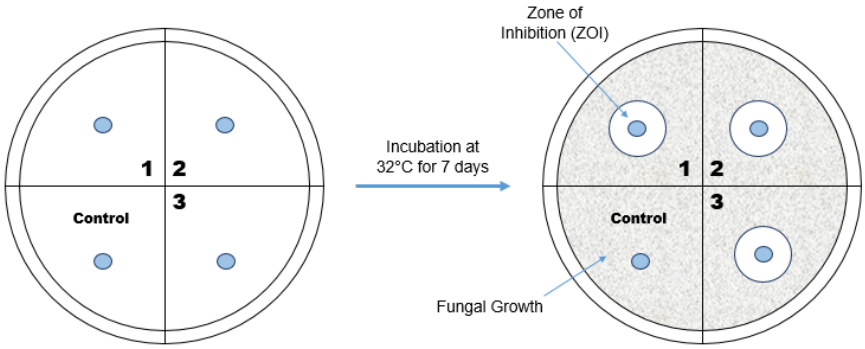

Figure 83. Schematic representation of disc diffusion assay (compiled by author).

Combination drug testing

For combination drug testing, the same procedure applied as above, however, 20µL of drug A and 20µL of drug B was placed onto the disc for a total of 40µL for each disc. 40µL of methanol was used as solvent control.

Results for both single and combination drug testing for disc diffusion, were obtained by measuring the radius of the zone of inhibition (from the edge of the disc to the edge of the zone), at three different points using a ruler, after 7

days after incubation. The final resulting value is expressed as the mean of the three measures obtained.

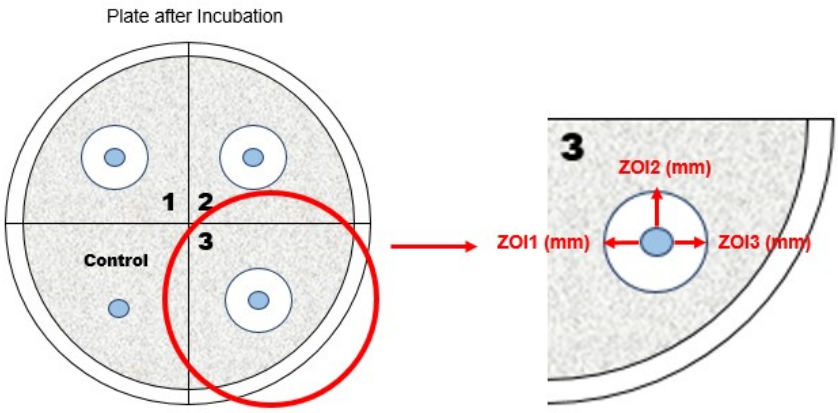

Figure 84. Diagram of the inhibition zone and the method of measure (compiled by author).

Broth microdilution susceptibility test

Broth microdilution susceptibility methodology was obtained from Clinical and Laboratory Standards Institution (CLSI), to determine minimum inhibitory concentration of antifungal drugs. This method requires the fungi to be in liquid medium.

Single drug testing

The broth microdilution susceptibility test was performed using sterile 96 well U bottom plate (Greiner bio-one, Cellstar, E181139A). 100µL of RPMI-1640 was transferred into wells of column 2-11 rows A-H of the plates, using a multichannel pipette. 200µL of antifungal drug (dissolved in methanol, which had been diluted at least 1:100 in RPMI) were transferred into wells of Column 1, rows A-G. A two fold dilution was performed by transferring 100µL of stock solutions from column 1 rows A-G, to column 2 rows A-G using a multichannel pipette. This procedure was repeated until column 10 rows A-G, where 100µL of solution was discarded to ensure all wells had a consistent volume.

100µL of inoculum was transferred into wells of column 11 to perform as growth control. Column 12 was used as sterility control with 200µL of RPMI only. 100µL of methanol in several concentration (1%; 0.1% and 0.01%) with RPMI was added into row H columns 1-10 which performed as solvent control. Finally, 100µL of inoculum was added into wells of Column 1-10 rows A-H.

The 96 well plate was incubated at 32°C and was visually observed after 7 days.

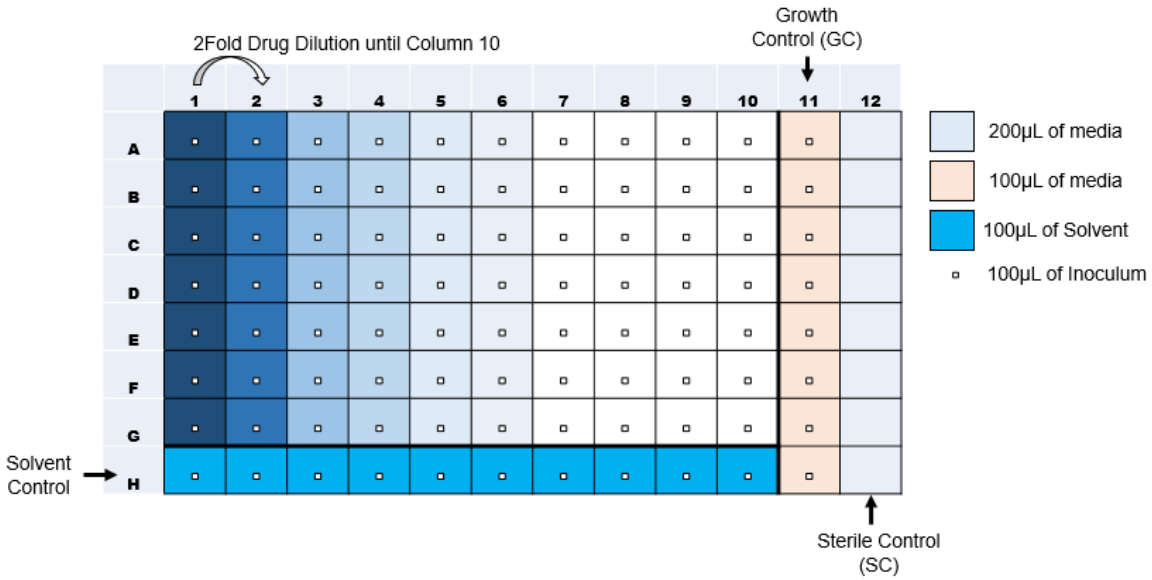

Figure 85. Schematic representation of a drug alone plate design for broth microdilution assay

Combination drug testing

To analyse the drug combination effects on the growth of T.Rubrum, checkerboard assay method was performed. This required preparation of drug A on plate 1, preparation of drug B on plate 2 and preparation of combining both drugs on plate 3.

Preparation of plate 1: Drug A

On a U bottom 96 well plate, 100µL of RPMI was transferred in wells of column 1-10 rows B-F using a Gilson pipette. This was followed by 200µL of drug A (dissolved in methanol, which had been diluted at least 1:100 in RPMI) in only row A columns 1-10. A two-fold dilution (from top to bottom) was performed using a multichannel pipette from row A to row B, then to row C, all the way down to row F, from which 100µL of the mixture was discarded to ensure all wells consisted of the same volume.

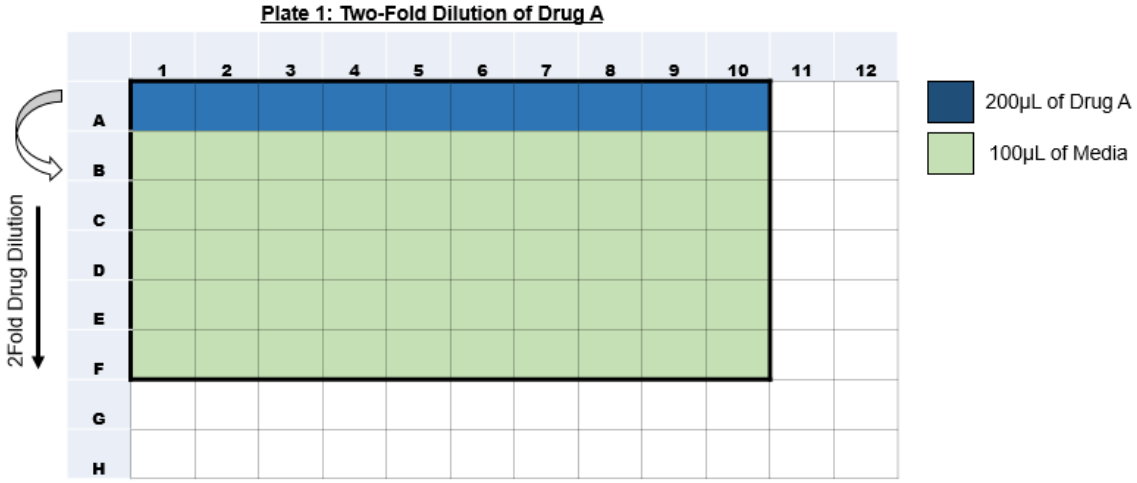

Figure 86. Schematic representation of Plate1

Preparation of plate 2: Drug B

On a U bottom 96 well plate, 100µL of RPMI was transferred in wells of columns 2-9, rows A-G. 200µL of drug B (dissolved in methanol, which had been diluted at least 1:100 in RPMI) was transferred in wells of column 10, rows A-G. A two-fold dilution (from right hand side of the plate to its left hand side) was performed using a multichannel pipette from wells of column 10 to column 9, then column 9 to column 8, up until column 2, from which 100µL of the mixture was discarded.

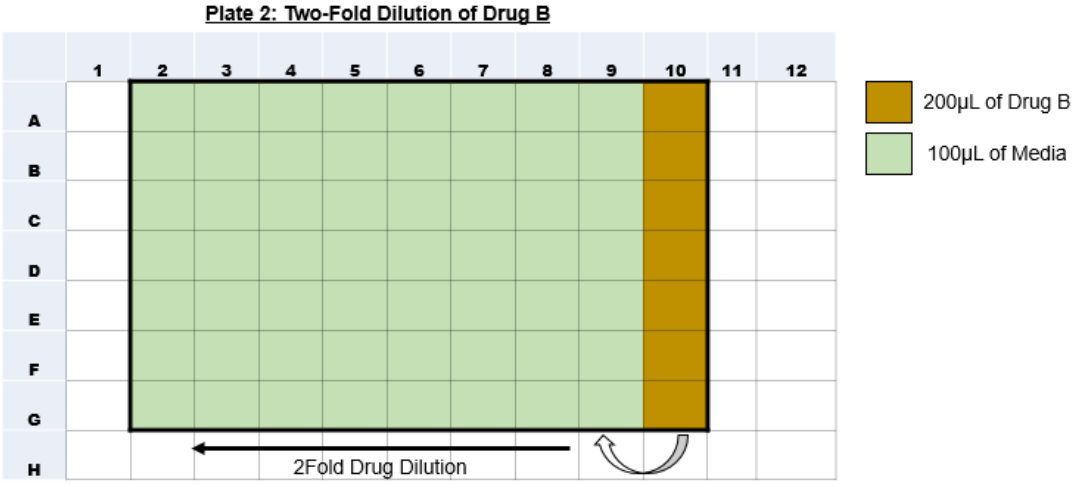

Figure 87. Schematic representation of Plate2

Preparation of third plate: combination plate

100µL of drug A was transferred from wells of column 1 rows A-F, from Plate 1 to exact same wells of plate 3. 100µL of drug B was transferred from wells of row G columns 2-10 from plate 2 to the exact same wells on plate 3. Column 1 row G contained 100µL of RPMI.

50µL of drug A from wells of columns 2-10 between rows A-F was transferred from plate 1 onto the exact same wells of plate 3. Similarly, 50µL of drug B from wells of column 2-10 rows A-F was transferred from plate 2 onto the exact same wells of plate 3.

200µL of RPMI-1640 was placed in column 12 rows A-H (sterility control column) of plate 3. 100µL of RPMI-1640 was placed in column 11 rows A-H (growth control column) of plate 3 and 100µL of methanol with RPMI in different concentration (as for single drug testing) was placed in wells of row H columns 1-10 (solvent control).

Finally, 100µL of prepared T.Rubrum inoculum was added into wells of column 1-11, rows A-G. This combination plate was incubated at 32°C and visually analysed after 7 days.

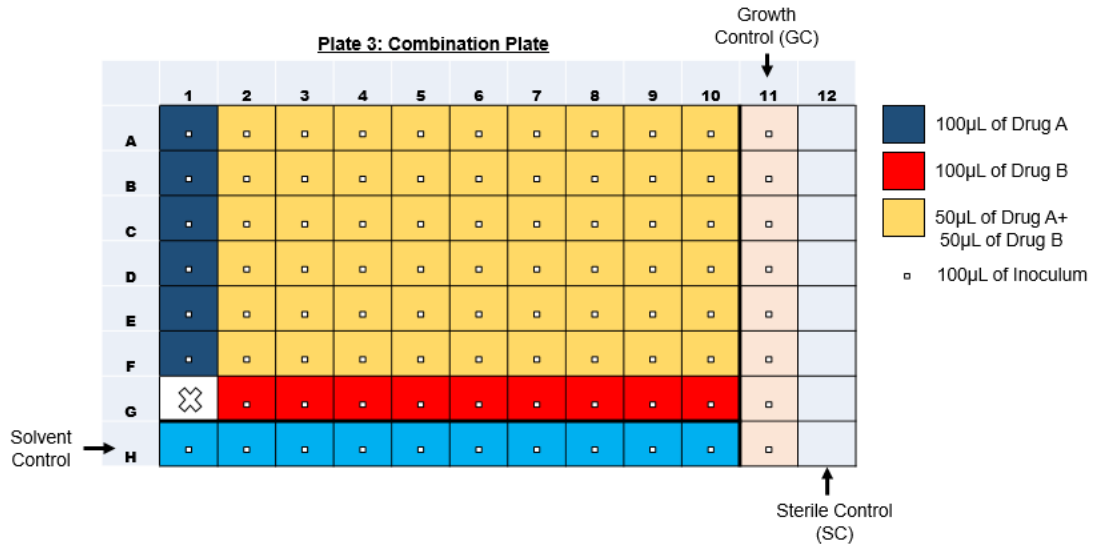

Figure 88. Schematic representation of Plate3

Interpretation of Results

Disc diffusion assay

As mentioned above, results for both single and combination drug testing for disc diffusion, were obtained by measuring the radius of the zone of inhibition

(from the edge of the disc to the edge of the zone), at three different points using a ruler, after 7 days after incubation. The final resulting value is expressed as the mean of the three measures obtained. A higher ZOI corresponds a higher potency of the drug. However, since the variability could be high, I decided to express ZOI (when 100% clear) as a range: from the smallest value (obtained by calculating the mean of the three radius of the zone of inhibition of one disc) to the highest. When the mean radius value was above 10mm, or when the zone of inhibition was so wide that it was not possible to measure 3 radius values, the results has been approximated as >10.

Broth microdilution assay

The influence of drug combination was determined by using the fractional inhibitory concentration index (FICI). This was to determine whether a synergistic, additive, indifferent or antagonistic effect is produced when combining one or two antifungals together. The equation below was used to calculate FICI (Meletiadis et al., 2009):

FIC of Drug A = MIC of drug A in combination with Drug B / MIC of drug A alone

FIC of Drug B = MIC of drug B in combination with Drug A / MIC of drug B alone

FICI = FIC of Drug A + FIC of Drug B

The values indicate: synergism (FICI ≤ 0.5); additive effect (0.5 > FICI ≤ 1); indifferent (1 > FICI ≤ 4) and antagonistic effect (FICI > 4).

Among the different methods of evaluation of the MIC of a drug in combination, I chose to follow the method described in the Clinical Microbiology Procedures Handbook (Anon, 1992), in which it is selected the lowest FIC index of all the non-turbi wells along the turbidity/non-turbidity interface.

Results and Discussion

Disc Diffusion

Disc diffusion experiments were conducted using several concentrations of the three antifungal drugs alone and in combinations as it is shown in the tables below.

Table 60. Summary of Disc Diffusion Assay results for drugs alone

Antifungal drug	Amount on disc (µg)	n	PROTOCOL	Comments
Terbinafine HCl	5	3	A	No Growth
	2.5	3	A	No Growth
	0.005	3	A	Methanol alone has a strong impact on fungal growth
	0.0025	3	B	Control affects results
Amorolfine HCl	5	3	A	No Growth
	2.5	3	A	No Growth
	0.01	3	A	Methanol alone has a strong impact on fungal growth
	0.005	3	B	Control affects results
Ciclopirox olamine	5	3	A	No Growth
	2.5	3	A	No Growth
	10	3	A	Methanol alone has a strong impact on fungal growth
	5	3	B	Control affects results

Table 61. Summary of Disc Diffusion Assay results for drug combinations

Antifungal drug	Amount on disc (µg)	n	Comments
Terbinafine HCl + Amorolfine HCl	T5 + A5	3	No Growth
	T2.5 + A2.5	3	No Growth
	T0.005 + A0.01	3	Methanol alone has a strong impact on fungal growth
Ciclopirox olamine + Terbinafine HCl	C5 + T5	3	No Growth
	C2.5 + T2.5	3	No Growth
	C10 + T0.005	3	Methanol alone has a strong impact on fungal growth
Amorolfine HCl + Ciclopirox olamine	A2.5 + C2.5	3	No Growth
	A0.01 + C10	3	Methanol alone has a strong impact on fungal growth

For the first experiments, both with drug alone and in combination, stock solution with a concentration of 250µg/mL and 125µg/mL were used for all the three drugs tested, to obtain a total amount of drug on the assay disc of 5µg or 2.5µg. In both cases, the concentrations resulted too high for Terbinafine HCl and Amorolfine HCl, inducing a complete fungal growth inhibition through all the petri dish. The other way round for Ciclopirox Olamine, for which the concentration seemed to low to induce a visible inhibition zone around the disc.

In the next experiment, drug concentrations were then lowered for T and A, while an higher concentration has been used for C. However, the results showed a strong impact of methanol alone on fungal growth, as visible in the figures below.

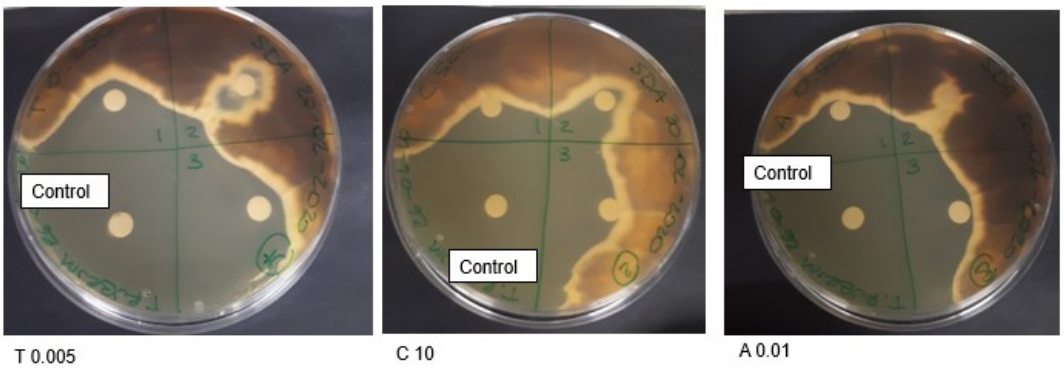

Figure 89. Disc Diffusion assay of drug alone against T. Rubrum. The methanol fungal growth inhibition is clearly visible.

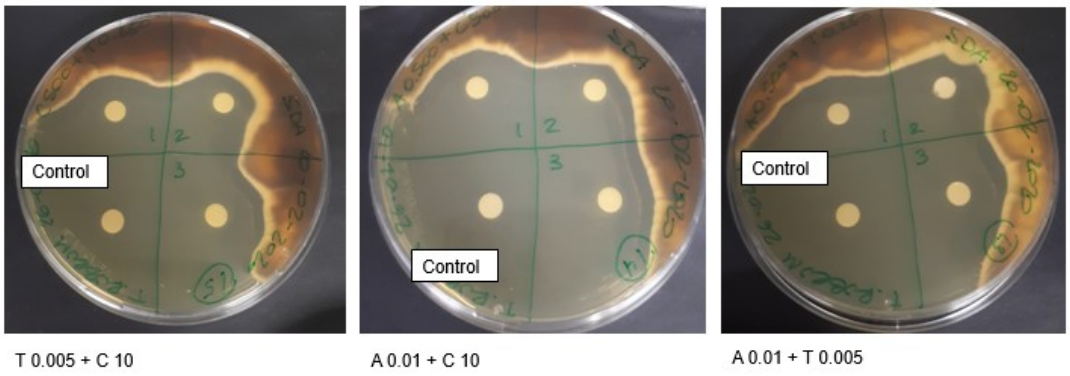

Figure 90. Disc diffusion assay of drug combination against T. Rubrum with clear growth inhibition induced by methanol alone.

To better understand methanol influence on fungal growth, the assay protocol has been changed to option B (10µL of prepared stock solution + 10µL of methanol onto each disc and 20µL of methanol as solvent control). Moreover, a test with different amount of methanol alone has been performed, to establish methanol minimum inhibitory amount.

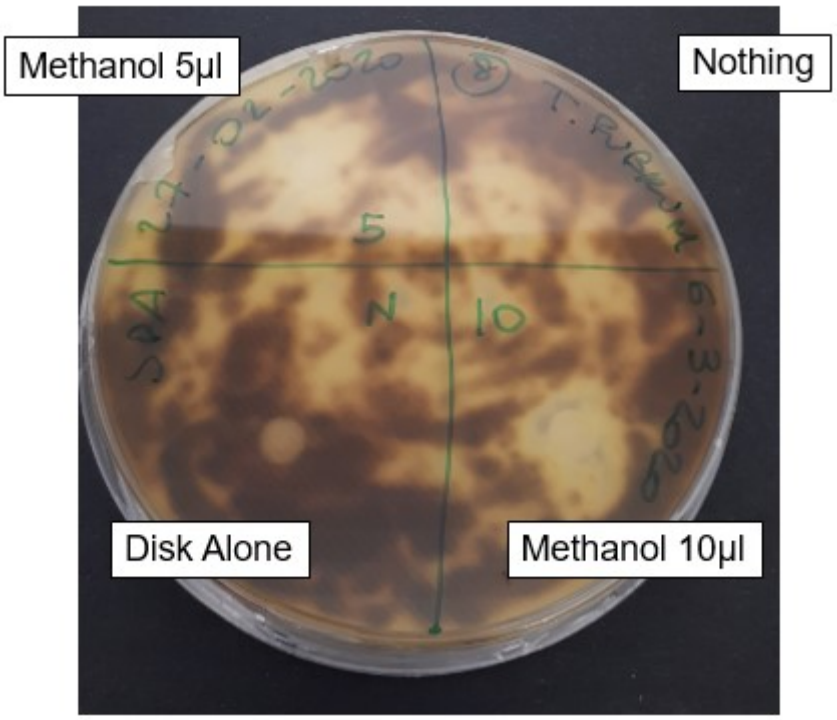

Figure 91. Disc Diffusion Assay with methanol alone against T. Rubrum

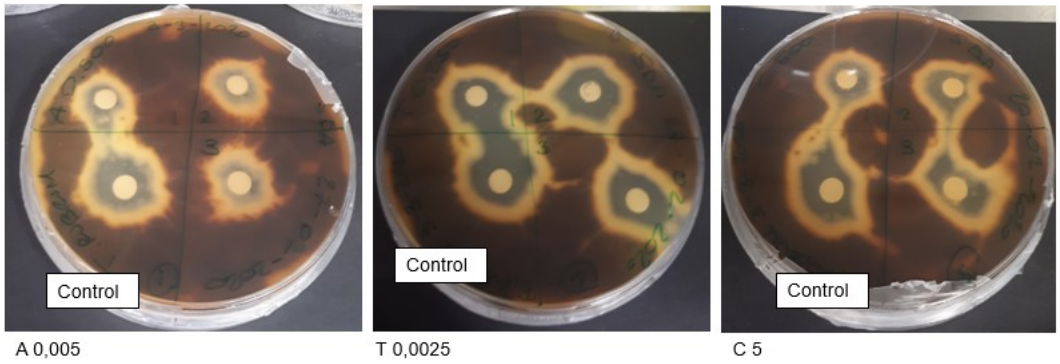

Figure 92. Disc Diffusion assay with drug alone against T. Rubrum (protocol B)

From the results obtained, the limit amount of methanol that can be used to have no inhibition of T. Rubrum growth is 5µL.

The next steps would be to repeat the experiments using 5µL as total amount:

- For drug alone assay: 5µL of prepared stock solution onto each disc and 5µL of methanol as solvent control;
- For drug combinations: 2,5µL of prepared stock solution of Drug A + 2,5µL of prepared stock solution of Drug B and 5µL of methanol as solvent control.

An alternative option could be to change solvent and use DMSO to prepare drugs stock solutions. However, the solubility rate of the selected antifungal drugs could be a limiting step.

The experiments have been repeated using a total amount of 5 µL for each disc and waiting at least 30-45 minutes between the loading of the drug solutions and the actual experiment.

In the table below are reported the results obtained. Plates where the control disc (methanol only) induced a fungal growth inhibition were not considered valid and the results were left out. The zone of inhibition (ZOI) is expressed as a range when the inhibition was 100% clear: from the smallest value (obtained by calculating the mean of the three radius of the zone of inhibition of one disc) to the highest. When the mean radius value was above 10mm, or when the zone of inhibition was so wide that it was not possible to measure 3 radius values, the results has been approximated as >10. In some cases ZOI was visible but no 100% (picture X).

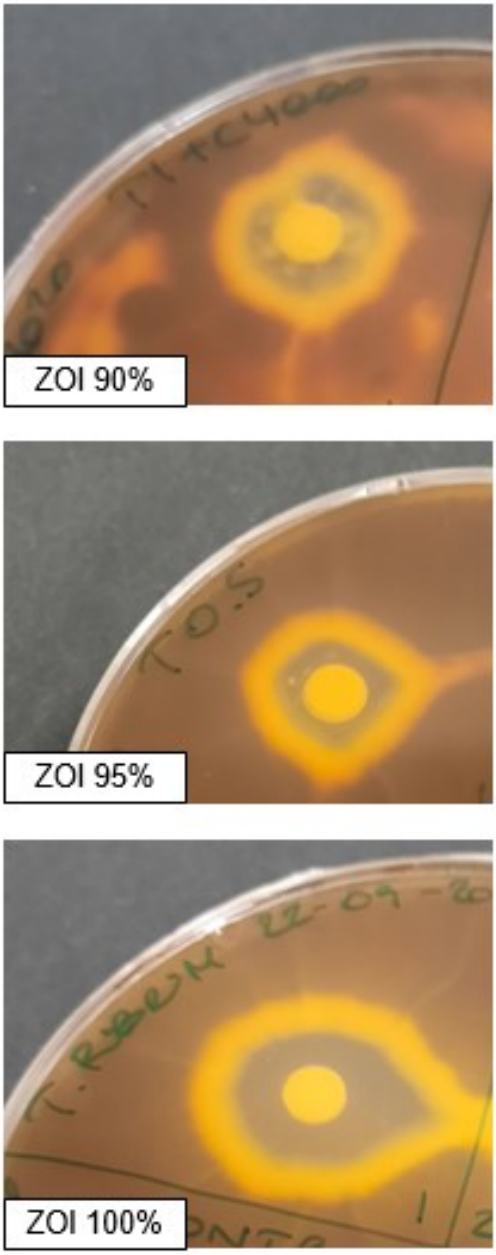

Figure 93. Visual comparison of different % of zone of inhibition.

Table 62. Disc Diffusion Results Summary.

Antifungal drug	Amount (µg)	n (n plates)	ZOI (radius range, mm)
Terbinafine HCl	0.0025	12 (4)	90% ZOI always visible
	0.005	12 (4)	2.3 - >10
	0.01	3 (1)	>10
Amorolfine HCl	0.01	3(1)	ni
	0.02	12 (4)	ni - 5 (90% ZOI)
	0.04	12 (4)	ni - >10
Ciclopirox olamine	10	3 (1)	ni
	20	2 (6)	ni
Terbinafine HCl + Amorolfine HCl	0.00125 + 0.01	12 (4)	90% ZOI
	0.00125 + 0.02	3 (1)	5 - >10
	0.0025 + 0.01	15 (5)	90% - 5.3
	0.0025 + 0.02	6 (2)	3.3 - >10
	0.005 + 0.01	6 (2)	6 - >10
	0.005 + 0.02	3 (1)	6.7 - >10
Terbinafine HCl + Ciclopirox olamine	0.00125 +10	9 (3)	ni
	0.0025+10	9 (3)	ni - 5
Amorolfine HCl + Ciclopirox olamine	0.01 + 10	9 (3)	ni
	0.02 + 10	9 (3)	ni - 6.3

Even with a discrete variability in the results, Terbinafine HCl (T) is confirmed as the most efficient as a single drug among the ones tested, followed by Amorolfine HCl (A) and Ciclopirox Olamine (C), which showed no inhibition at the tested concentrations. While T+C and A+C combinations did not show any relevant increase in antifungal effect, T+A seems a promising option.

In fact, a ZOI (90%) is already visible with a combination of 0.00125µg+0.01 µg of T and A respectively and the effect is even more clear with a T+A combination of 0.0025 µg + 0.01 µg (ZOI range between 90% and 100% with a mean radius of 5.3mm). In comparison, the same amount of the two drugs alone showed a lower antifungal effect, with no inhibition of fungal growth for 0.01 µg of A alone and a 90% ZOI for T alone. At the opposite, the efficiency of the other combinations seemed not relevant.

Broth Microdilution

Broth microdilution experiment were conducted and visually observed after 7 days of incubation. Single drug testing and combination drug testing against T. rubrum was performed to obtain MIC_{100} values. Experiments were repeated (n) for validation and geometric means were calculated.

Single Drug Testing

Single drug testing of antifungal drugs were performed and geometric mean MIC_{100} are reported in table 3. Out of the three antifungal drugs, Terbinafine HCl showed to be the most potent drug (3.57µg/mL), followed by Ciclopirox Olamine (4.36µg/mL) and Amorolfine HCl (11.76µg/mL).

Table 63. Geometric mean of MIC100 of drugs when used alone against T. Rubrum

***Trichophyton Rubrum* strain ATCC 28188**			
Antifungal Agent	**n**	**Geometric mean MIC_{100} (µg/mL)**	**Range of MIC_{100} in replicates (µg/mL)**
Terbinafine HCl	25	3.57	1.28->10.24
Ciclopirox Olamine	30	4.36	4-8
Amorolfine HCl	22	11.76	10.24-20.48

The ranges of MIC_{100} values were wide, in particular for Terbinafine HCl. In order to understand the possible cause of the high variability of the results the MIC_{100} data were grouped based on the day of preparation of the different drug dilutions.

Table 64. Timetables of the different operations performed before the actual experiment and related mean Terbinafine HCl MIC_{100}.

Terbinafine HCl								
Stock Solution in Methanol	05-feb	17-feb	17-feb	17-feb	17-feb	17-feb	17-feb	17-feb
1st dil 1:100 in RPMI	12-feb	17-feb	25-feb	25-feb	25-feb	05-mar	10-mar	12-mar
2nd dil 1:100 or 1:10 in RPMI	-	18-feb	26-feb	28-feb	03-mar	06-mar	11-mar	13-mar
Experiment	12-feb	19-feb	26-feb	28-feb	03-mar	06-mar	11-mar	13-mar
n	3	5	2	2	7	2	2	2
Geometric mean MIC_{100} (µg/mL)	**1,28 ± 0**	**4,46 ± 1,14**	**2,56 ± 0**	**>10,24**	**10,24 ± 0**	**1,28 ± 0**	**1,28 ± 0**	**2,56 ± 0**

As it is possible to observe form table 2, while the second dilution with RPMI has been usually performed the same day as the experiment, the first one has

been done at least one day before, in order to allow the complete dissolution of the drug overnight. However, Terbinafine does not seem stable in RPMI, since using the same dilution for the following experiments has led to a reduction in drug potency. Here below is reported a graph showing the correlation between Terbinafine HCl MIC_{100} values and the number of days passed from the first dilution of the drug and the actual experiment.

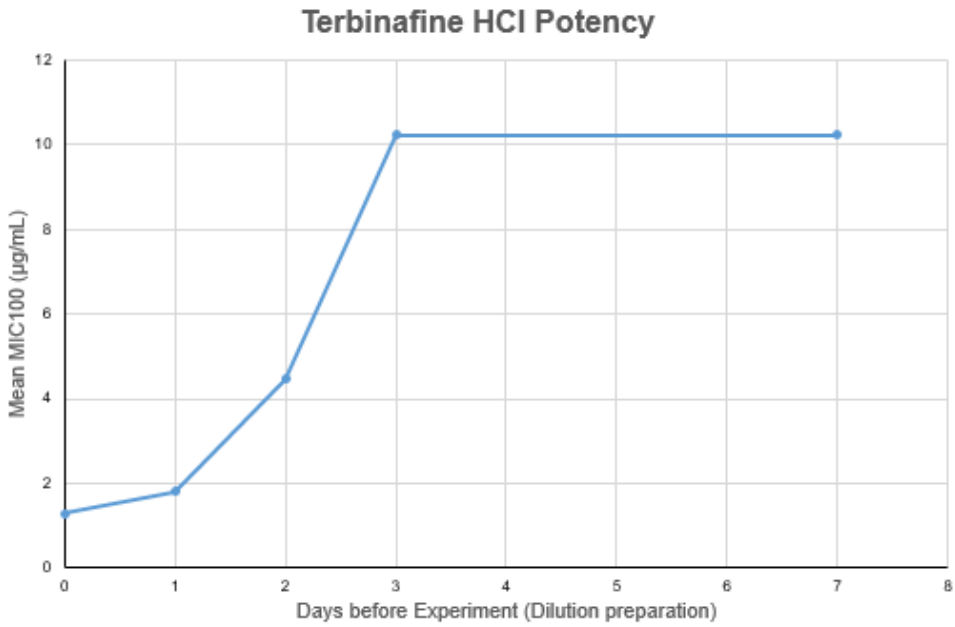

Figure 94. Graphic representation of Terbinafine HCl mean MIC_{100} values in function of the date of preparation the first drug dilution.

With these considerations, I have decided to keep as valid only terbinafine HCl data of the experiments with the first dilution performed at most 1 day before the actual experiment day, all the other data need to be excluded. Amorolfine HCl and Ciclopirox Olamine did not show the same time-sensitivity. However, all the experiments where it was not possible to find a MIC_{100} value have not been considered in the calculations.

In the table below are reported the updated data after the selection. The data obtain for single drug experiments have been divided from the one of the drug alone obtain inside a combination plates. Then a general geometric mean has been calculated along with the standard deviation.

Table 65. Mean MIC_{100} of the three antimycotic drugs alone: comparison between data obtained in single drugs plates and drug combinations plates.

***Trichophyton Rubrum* strain ATCC 28188**					
Antifungal Agent	**n (drug alone plates)**	**Geometric mean MIC_{100} (µg/mL)**	**n (drug combination plates)**	**Geometric mean MIC_{100} (µg/mL)**	**MIC_{100} Total Mean (µg/mL)**
Terbinafine HCl	5	1.28±0	6	2.03±0.60	1.65±0.65
Ciclopirox olamine	24	4±0	8	5.66±2.00	4.36±1.34
Amorolfine HCl	4	10.24±0	6	12.90±4.84	11.76±4.32

Looking at the total geometric mean of the MIC_{100} values, Terbinafine HCl has been confirmed as the most potent drug (1.65µg/mL), followed by Ciclopirox Olamine (4.36µg/mL) and Amorolfine HCl (11.76µg/mL).

Combination Drug Testing

The results of the combination drug testing are still initial and more replicates must be performed. In the tables below are reported all the data from the preliminary experiments.

Table 66. Summary of the combination plates data using Terbinafine HCl + Amorolfine HCl against T.Rubrum.

Terbinafine HCl + Amorolfine HCl			
	Exp 26-2 MIC_{100} (µg/mL)	Exp 11-3 MIC_{100} (µg/mL)	Exp 13-3 MIC_{100} (µg/mL)
T alone	2.56	1.28	2.56
T with A	2.56	1.28	1.28
A alone	10.24	10.24	20.48
A with T	0.32	0.64	10.24
FIC of T	1.000	1.000	0.500
FIC of A	0.031	0.063	0.500
FICI	1.031	1.063	1.000
n	**3**		
FICI Mean	**1.031**		

Table 67. Summary of the combination plates data using Ciclopirox Olamine + Amorolfine HCl against T.Rubrum.

Ciclopirox Olamine + Amorolfine HCl				
	Exp 26-2 MIC_{100} (µg/mL)	Exp 28-2 MIC_{100} (µg/mL)	Exp 11-3 MIC_{100} (µg/mL)	Exp 13-3 MIC_{100} (µg/mL)
C alone	4	4	8	8
C with A	8	8	8	16
A alone	10.24	11.76	10.24	20.48
A with C	0.04	0.04	0.04	0.08
FIC of C	2.000	2.000	1.000	2.000
FIC of A	0.004	0.003	0.004	0.004
FICI	2.004	2.003	1.004	2.004
n	**4**			
FICI Mean	**1.686**			

Table 68. Summary of the combination plates data using Terbinafine HCl + Ciclopirox Olamine against T.Rubrum.

Terbinafine HCl + Ciclopirox Olamine			
	Exp 26-2 MIC_{100} (µg/mL)	Exp 11-3 MIC_{100} (µg/mL)	Exp 13-3 MIC_{100} (µg/mL)
T alone	2.56	1.28	2.56
T with C	0.04	0.04	1.28
C alone	4	8	8
C with T	4	16	8
FIC of T	0.016	0.031	0.500
FIC of C	1.000	2.000	1.000
FICI	1.016	2.031	1.500
n	**3**		
FICI Mean	**1.457**		

Overall, all the three drug combinations seem to have an indifferent effect with a FICI > 1. In particular for the combination T+C the mean FICI=1.457 and for A+C and the mean FICI=1.686. The combination of Terbinafine HCl with Amorolfine HCl seems the more promising one, with a mean FICI=1.031. All the experiments must be repeated to have more reliable data.

Conclusion

This work aimed to assess the efficacy of drugs alone (Terbinafine HCl, Amorolfine HCl and Ciclopirox Olamine) and in combination against

Trichophyton Rubrum. The experiments were carried out using two different assays, broth microdilution and disc diffusion, in order to understand the applicability of these assays to dermatophytes and to compare the results with the two techniques. In fact, the above mentioned assays are standardized techniques for antifungal drugs assessment against other fungi species, but there is still no standardized protocols when considering dermatophytes.

Overall, the methods chosen have demonstrated to be suitable for *T. rubrum* testing. However, two main criticalities emerged that must be taken into account to optimize the protocol:

- Terbinafine HCl showed to be sensitive to degradation or to a loss in solubility degree when in aqueous media like RPMI: timing in drug solutions preparation is then crucial in order to have reliable data for broth microdilution assay.
- *T. rubrum* solid agar growth has shown to be strongly affected by methanol alone, which is the best option as a solvent to be used to dissolve all the drugs chose for this study. The amount of drug solution to be loaded on the discs and the waiting time between loading and positioning of the disc on the agar need to be carefully considered.

Terbinafine HCl has shown to be the most effective among the drugs selected for the assessment with both the techniques, while Amorolfine HCl and Ciclopirox Olamine gave a different response. According to the results obtained with broth microdilution Ciclopirox Olamine seems more effective than Amorolfine HCl; disc diffusion results, on the contrary, showed a higher potency of Amorolfine HCl in comparison to Ciclopirox.

The results obtained for drug combinations with the two techniques are comparable since both of them showed T+A combination as the most promising one.

PROTOCOL SET-UP FOR THE EVALUATION OF N-METHYL-SPERMIDINE NAIL PERMABILITY USING A NOVEL DUAL-FLOW BIOREACTOR

The aim of this study was the assessment of the trans-ungual permeability of N-methyl-spermidine through an “in vitro” study using keratin membranes obtained from bovine hooves as a model. The product was tested dissolved in two different vehicles, glycolic acid (BE0601) and urea (BE0602) in order to understand how solution pH could affect the permeation through the membranes. As diffusion cells, LiveBox 2® silicon diffusion cells (IVtech, Italy) were selected [97].

Materials and Methods

Production and selection of bovine hoof membranes

Freshly slaughtered bovine hooves were dipped in liquid nitrogen, cored according to the chosen diameter by using a Plug cutter Rolson® with 16 mm diameter, Rolson Tools LTD (Twyford, United Kingdom)**,** and cut in slices with a precision lathe Graziano SAG 12 (Tortona, Italia)**,** equipped with HSS blade (high-speed-steel), Freud® S.p.A. (Tavagnacco, Italia) and an analog Centesimal Comparator with magnetic support Borletti®, LTF S.p.A. (Antegnate, Bergamo).

Before the experiment the membranes were sanitized with a sequence of washing with Ethanol 70% v/v and a Benzalkonium Chloride mixture [0,4 g Benzalkonium Chloride (Farmalabor); 70 g isopropyl alcohol (Carlo Erba); distilled water to 100g] and then maintained in a climatic chamber at 25°C and 40% R.H (ClimaCell 111 MMM).

After production,15 membranes with a thickness of about 180µm and 8 membranes with a thickness of about 400µm were selected.

All the membranes used for the permeability study were characterized using the Nail StrainStress Meter NM100 (Courage & Khazaka, Cologne, Germany) to calculate thickness [86].

Density was calculated dividing the membrane weight for its volume (mg/mm^3).

The morphological evaluation has been performed through the acquisition of the image by a digital microscope to assess the surface desquamation features.

Diffusion Cells

LiveBox 2® silicon diffusion cells (IVtech, Italy) were selected for the evaluation of the trans-ungual permeability. These cells were used with a tangential flow configuration and are equipped with two compartments (acceptor and donor) able to simulate the physiological barrier conditions. In particular, the apical and basal chamber are perfused by two independent flows which have a fluidic recirculation system. The system is equipped with a peristaltic pump.

Between the two compartments of the LiveBox 2® system, a membrane obtained from a 3 year old Chianina bovine hoof with two different thicknesses was placed. All the membranes used were characterized by morphology, thickness and mechanical features before the experiments.

Experimental Procedure

The study was divided into two parts:

1. preliminary phase in which two types of membranes with a thickness of about 200 µm [experiment A (180 ± 30 µm)] or 400 µm [experiment B (400 ± 50 µm)] were investigated using both products (BE0601 and BE0602) in order to verify any criticality;
2. data confirmation phase in which membranes with a thickness of about 200 µm were tested with the BE0601 product under optimized experimental conditions [experiment C (180 ± 30 µm)].

The study was carried out according to the following experimental procedure:

- Production and characterization of the membranes.
- Preparation of air/liquid interface diffusion cells. The donor compartment is exposed to air while the acceptor compartment differs for experiment A, B, C as follows: it contains water (experiment A) or it contains 1% phenoxyethanol water solution.
- Application of 10 µL of product (BE0601 or BE0602) for 8 times each 48 hours.
- Washing of the donor compartment every 24 hours using a 0.2% solution of a non-ionic surfactant followed by rinsing for 5 minutes to simulate the daily washing conditions.

The experiments were performed keeping all the instruments in a climatic chamber at a temperature of 32 ± 2 °C and a relative humidity of 60 ± 5%. For each experiment, the cells used were classified as A1, A2 ... B1, B2 ... One cell was used as a control for the membrane interference in which an equal volume of fluid was applied in as the acceptor medium. A second cell was used as a control for the active principle in which an equal volume of active principle dissolved in the fluid used as the acceptor medium was applied. In experiments A and B (preliminary phase), the BE0601 product was applied in 3 cells and the BE0602 product was applied in other 3 cells. In the experiment C (data confirmation phase), 6 cells were used in which only the product BE0601 was applied.

The solution in the acceptor compartment was measured and collected after the second application and at the end of the experiment (after 8 applications). The samples collected were analyzed by HPLC MS-MS for the quantitative analysis of the active principle.

Quantitative analysis of the active principle

The N-methyl-spermidine was determined using HPLC-MS/MS system.

The internal standard solution was prepared dissolving an appropriate amount of rolipam in dimethyl sulfoxide (DMSO) to give 1mg/ml stock solution stored at 4°C. 1mg/ml solution was then diluted to obtain 100ng/ml solution usig 50% acetonitrile in water MilliQ. For the preparation of the sample the following procedure has been apllied: 50µL of samples (phosphate buffer saline or methanol) were added to 50µL of PBS or MeOH in order to have the same components across samples. Samples were then added with 200 µL of water/acetonitrile (50/50 v/v) containing rolipram as internal standard (100ng/mL) and then injected on to an HPLC-MS/MS system. A calibration curve was prepared and analysed together with unknown samples in order to determine their concentration.

In the tables below are reported the analytical conditions used.

Table 69. Chromatographic Conditions

Autosampler	CTC HTS PAL
Injection Wash Solvent 1	50/50 Water/Acetonitrile (v/v) (0.1% Formic Acid)
Injection Wash Solvent 2	50/50 Water/Acetonitrile (v/v) (0.1% Ammonia)
Dilutor Wash Solvent	100% Acetonitrile
Injection Volume	10µL
Chromatography System	Agilent 1100 binary solvent delivery HPLC System
Column	Synergi MAX-RP column (30mm x 2.0mm, 4µm particle size)
Column Temperature	Room Temperature
Run Time	1.5 minutes
Mobile Phase A	100% water (0.1% HeptaFluoroButyric Acid)
Mobile Phase B	100% acetonitrile (0.1% HeptaFluoroButyric Acid)
Flow Rate	1.5 mL/min

Table 70. Mass Spectrometer Conditions

MS	API4000 (AB Sciex)™
Ion source	TurboIonSpray™ at 600°C
Gas 1 Setting	40 psi
Gas 2 Setting	50 psi

Contact angle

At the end of the study, an assessment of the superficial state of the membranes was performed by evaluating the contact angle (θ). The direct method was used to directly visualize the phenomenon that occurs at the interface between the water drop and the sample. By measuring drop base and height using simple trigonometric formulas, the contact angle is obtained using the CoolinTech 2.0 software.

Results and Discussion

Preliminary phase

Experiment A

Table 71 shows the results regarding the characterization of all the membranes involved in the study.

Table 71. Characterization of the membranes of the experiment A

	Sample	*Membrane code*	*Thickness* (μm)	*Density* (mg/mm^3)
Exp A1	BE0601_A1_1	281	182	0,80
	BE0601_A1_2	282	180	0,79
	BE0601_A1_3	289	179	0,77
	Control	296	182	0,82
Exp A2	BE0602_A2_1	279	183	0,75
	BE0602_A2_2	280	185	0,79
	BE0602_A2_3	290	194	0,82
	MS 1%	291	200	0,75

As shown in Table 71, the membranes of experiment A have homogeneous characteristics as for thickness and density, while they show some differences correlated with the morphological features. For example, Figure 95 shows the images of the membrane 281, characterized by smooth morphology, and of the membrane 289 characterized by the highly desquamated surface.

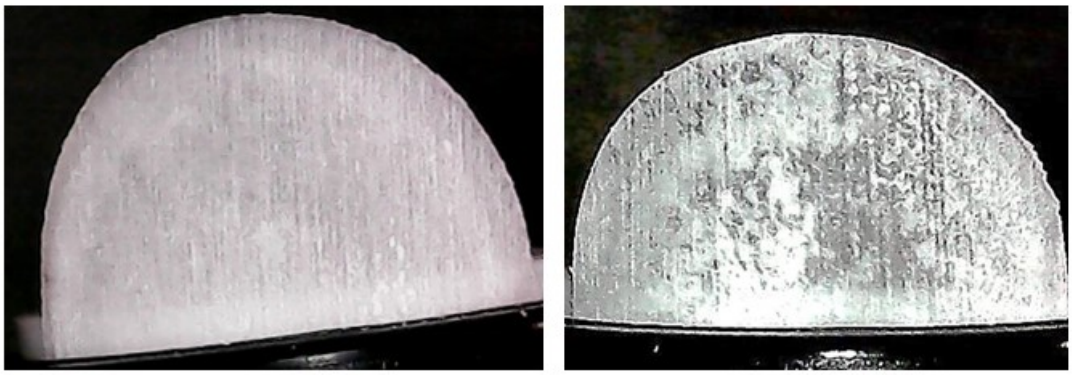

Figure 95. Digital image of membrane 281(on the left) and 289 (on the right).

Table 72 shows the results expressed as % active permeated after 2 and 8 applications. The theoretical concentrations were calculated considering the amount of fluid present in the acceptor compartment.

*Table 72. Results of experiment A: T=theoretical data; E=experimental data; *=mould presence in the sample; bql=below quantification limit*

		ng/mL of MS after 2 applications		*ng/mL of MS after 8 applications*		*Permeated MS %*	
	Sample	*T*	*E*	*T*	*E*	2 appl	8 appl
Exp A1	BE0601_A1_1	37244	Bql	177383,6	31300	**4,78**	**10,5**
	BE0601_A1_2	45351	2170	217151,2	10800		
	BE0601_A1_3	44053	Bql	250000	22200		
	Control	0	Bql	0	bql	-	-
Exp A2	BE0602_A2_1	43011	Bql	173160	bql*	**14,34**	**14,11**
	BE0602_A2_2	42919	6710	216544	41500		
	BE0602_A2_3	47059	6140	199975	18100		
	MS 1%	49020	3520	198623	21500	**7,18**	**10,8**

The results obtained with the control membrane show that there are no interferences in the quantitative analysis of N-methyl spermidine, however the absence of the preservative in the acceptor compartment solution resulted in mold formation.

Nevertheless, the results show that the active substance seems to penetrate through the membrane in a greater way when in a basic solution medium after two applications but in similar way in all the products after 8 applications.

Experiment B

In this experiment, thicker membranes were used and 1% of phenoxyethanol was added to the water present in the acceptor medium in order to avoid contamination problems. Table 73 shows the results of the characterization of all the membranes involved in the study.

Table 73. Characterization of the membranes of the experiment B

	Sample	*Membrane code*	*Thickness* (μm)	*Density* (mg/mm^3)
Exp B1	BE0601_B1_1	300	440	1,11
	BE0601_B1_2	301	434	0,97
	BE0601_B1_3	303	434	1,04
	Control	310	452	0,94
Exp B2	BE0602_B2_1	304	410	0,94
	BE0602_B2_2	305	462	0,95
	BE0602_B2_3	311	424	0,86
	MS 1%	313	383	0,86

Figure 96 shows the images of the membrane 303, morphologically heterogeneous, and of the membrane 301, with a very smooth surface.

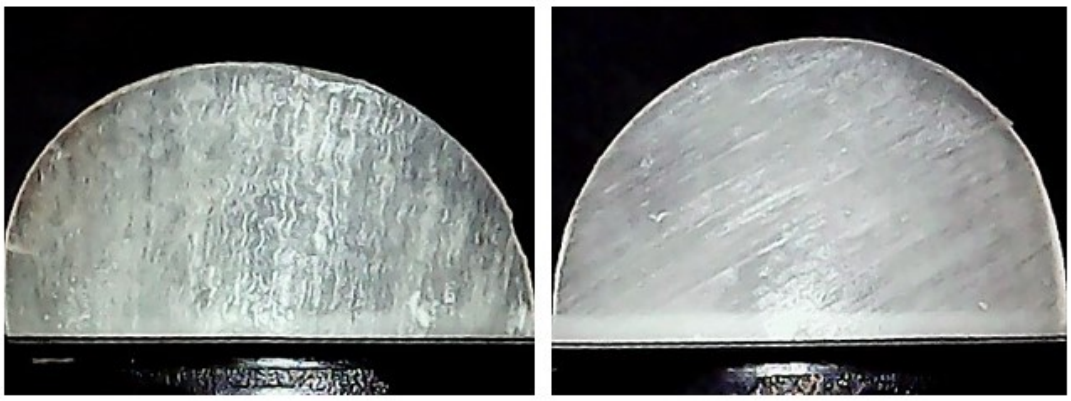

Figure 96. Digital image of membrane 303(on the left) and 301 (on the right).

Table 74 shows the results expressed as % active permeated after 2 and after 8 applications.

*Table 74. Results of experiment B: T=theoretical data; E=experimental data; *=not reliable; bql=below quantification limit*

		ng/mL of MS after 2 applications		*ng/mL of MS after 8 applications*		*Permeated MS %*	
	Sample	*T*	*E*	*T*	*E*	2 appl	8 appl
Exp B1	BE0601_B1_1	41667	20300	217175	92900	**39,56**	**39,01**
	BE0601_B1_2	44732	bql	203666	76600		
	BE0601_B1_3	45725	13900	198390	72700		
	Control	0	bql	0	bql	-	-
Exp B2	BE0602_B2_1	47203	4930	203317	32400	**10,92**	**45,64**
	BE0602_B2_2	72438	2040	199339	54200		
	BE0602_B2_3	42900	8360	135413	127000		
	MS 1%	48461	4410	170067	188000	**9,10**	**110,5***

Using the preservative in the acceptor compartment there were no contamination problems during the experiment. The results show that BE0601 allows a constant permeation of N-methyl spermidine throughout the experiment and in a comparable way among different membranes, while product BE0602 shows a non-homogeneous permeation of N-methyl spermidine throughout the experiment, with a dependence by the membrane used. The data concerning the complete permeation of N-methyl spermidine in water is not to be considered reliable because the aqueous solution, with zero viscosity, passed in the acceptor compartment through the membrane sides during the experiment.

After performing the two preliminary studies the product BE0601 has been selected to continue with the confirmation phase experiment.

Data confirmation phase

Experiment C

This last experiment was performed to confirm the permeation data obtained with the BE0601 product using membranes of about 180 µm thickness and, in order to avoid contamination problems, 1% of phenoxyethanol was added to the water present in the acceptor medium. Table 75 shows the results regarding the characterization of all the membranes involved in this study.

Table 75. Characterization of the membranes of the experiment C

Sample	*Membrane code*	*Thickness*(µm)	*Density* (mg/mm^3)
BE0601_C1_1	283	173	0,65
BE0601_C1_2	284	175	0,72
BE0601_C1_3	285	173	0,68
BE0601_C1_4	286	171	0,64
BE0601_C1_5	288	177	0,67
BE0601_C1_6	295	169	0,78
MS 1%	298	158	0,73

As shown in table 75, the membranes of experiment C have quite homogeneous characteristics as for thickness and density.

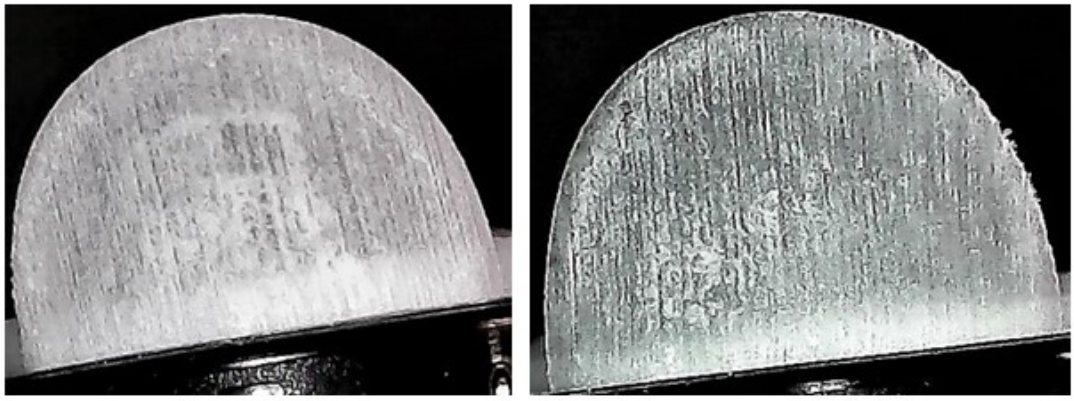

Figure 97. Digital image of membrane 295(on the left) and 283 (on the right).

Table 76 shows the results expressed as % active permeated after 2 and 8 applications.

*Table 76. Results of experiment C: T=theoretical data; E=experimental data; * during the first 2 applications, the cell often had bubbles in the acceptor compartment; **values are the mean of 3 samples (1,5,6) or 5 samples (1,2,3,5,6) respectively; bql=below quantification limit*

	ng/mL of MS after 2 applications		*ng/mL of MS after 8 applications*		*Permeated MS %*	
Sample	*T*	*E*	*T*	*E*	2 appl	8 appl
BE0601_C1_1	49875	5790	185390	88900	**31,62-42,62*****	**84,32**
BE0601_C1_2*	47619	56000	175233	176000		
BE0601_C1_3	40984	256	199092	201000		
BE0601_C1_4	40650	bql	206154	130000		
BE0601_C1_5	43011	18000	179075	161000		
BE0601_C1_6	43956	18200	172807	179000		
MS 1%	53333	15200	190026	83600	**28,50**	**44,00**

Results show that BE0601 product allows a passage of N-methyl spermidine of almost twice as much as the aqueous solution of the active. However, the values obtained with experiment C are greater than those obtained with experiment A: the reason could be the lower cdensity of the membranes used for experiment C since such membranes may mimic the less firmness of brittle nails in comparison to the healthy ones.

At the end of the study, an assessment of the superficial state of the membranes was performed by evaluating the contact angle (θ) (Figure 98).

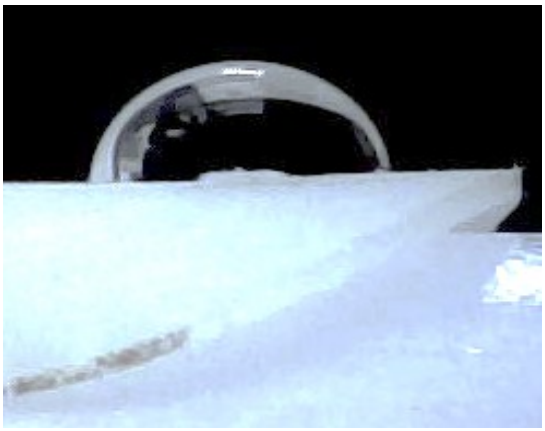

Figure 98. Digital image of the interface phenomenon between the water drop and the membrane

Table 77 shows the results of the membranes after the experiment C compared with the mean values obtained from the analysis of at least 5 untreated membranes (Control membranes).

Table 77. Contact angle values (experiment C)

Membrane	***Sample***	**Θ**
283	BE0601_C1_1	77,3
284	BE0601_C1_2	87,2
285	BE0601_C1_3	50,24
286	BE0601_C1_4	37,84
288	BE0601_C1_5	61,12
295	BE0601_C1_6	72,7
298	MS 1%	46,66
Control	-	45,8±8,57

Results show that the MS-treated membrane has a contact angle comparable to the control membranes, while most of the membranes treated with the tested product have a higher value, which could be indicator of product residue presence on the surface of the membrane, which modifies the membrane hydrophilic/lipophilic characteristics.

To summarize, the novel dual-flow bioreactor has been shown to be a suitable permeability evaluation system for bovine membranes of about 200µm thickness. The system allow a long term permeation study in continuum, with

controlled temperature and humidity during all the experiment. Moreover, the novel technology allows to mimic real-life washing condition during the assay. The product BE0601 (glycolic acid solution of the active) has shown the best permeability performance, since it allows the passage of about 40% of N-methyl spermidine through the membranes after two applications, which is almost twice as much as the aqueous solution of the same active. After 8 applications BE0601 allows the passage of about 80% of the active, which is almost twice as much as the % obtained with the aqueous solution of the active. In addition, the contact angle analysis showed how the product is able to adhere to membrane surface constituting a film that may be exert a protective effect in order to promote active efficacy.

Conclusion

The aim of the study was the assessment of the trans-ungual permeability of N-methyl-spermidine through an “in vitro” study using membranes obtained from bovine hoof characterized by different thickness. On the basis of the results described above, the novel in-vitro model protocol was effective in the evaluation of N-methyl spermidine permeability on membranes with a thickness of 200 μm. And the acidic vehicle seemed to be the most effective in promoting the N-methyl spermidine passage through the membranes.

A NEW PROTOCOL TO EVALUATE ANTI-ADHESIVE EFFECT AGAINST FUNGAL NAIL INFECTIONS

The aim of this work has been to develop a protocol to assess the ability of a product to prevent fungal adhesion on the nails, which is the first step of the infection. The novelty of this work is that of seeing not only the antifungal effect, which can be evaluated with a more traditional assay to quantify the MIC (Minimum Inhibitory Concentration) of the tested drugs, but also the anti-adhesive effect (AE) of a certain substance on the nail.

Bovine hoof keratin membranes have been chosen as in vitro model, since their wide used in several fields of nail related research [12] [13].

In order to evaluate the possible protective activity of products against fungal contamination on the nail plate, it was necessary firstly test the attitude of the hoof membranes to a fungal contamination without any product, using two specific and main representative fungi strains: *Trichophyton rubrum* and *Candida albicans*. Then, a positive and a negative control were tested by introducing the membranes (treated and not) into the fungal suspensions for three different times (15 min, 5 hours and 24 hours).

Materials and Methods

Membranes selection

All hoof membranes used for these experiments have been obtained from fresh slaughtered 3 years old cattle (Azienda Agricola Pluderi Marcellino, via Villa Serafina 19, San Colombano al Lambro).

Freshly slaughtered bovine hooves were dipped in liquid nitrogen, cored by using a Plug cutter Rolson® with 16 mm diameter, Rolson Tools LTD (Twyford, United Kingdom), and cut in slices of about 300µm with a precision lathe Graziano SAG 12 (Tortona, Italia).

Before every experiment and evaluation the membranes were sanitized with a sequence of washing with Ethanol 70% v/v and a Benzalkonium Chloride mixture [0,4 g Benzalkonium Chloride (Farmalabor); 70 g isopropyl alcohol (Carlo Erba); distilled water to 100g] and then maintained in a climatic chamber at 25°C and 40% R.H (ClimaCell 111 MMM).

The membrane morphology analysis was performed by a digital microscope model BW 1008 to evaluate the surface desquamation features. The membranes were classified based on their morphological superficial properties and selected as flaky or semi-flaky in order to have homogeneous groups.

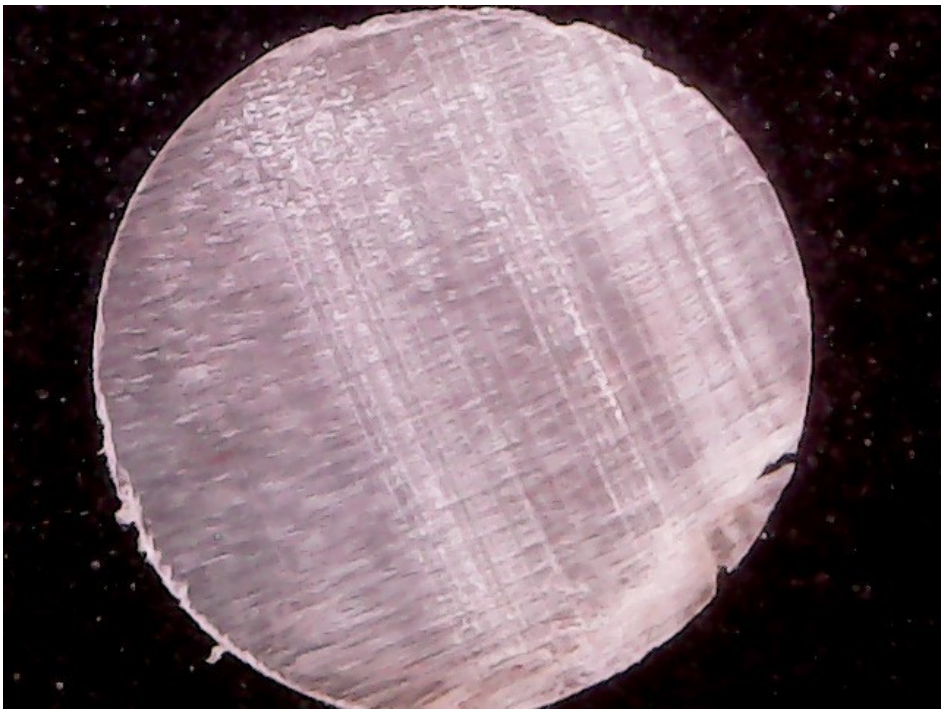

Figure 99. Semi-flaky membrane selected for the experiment.

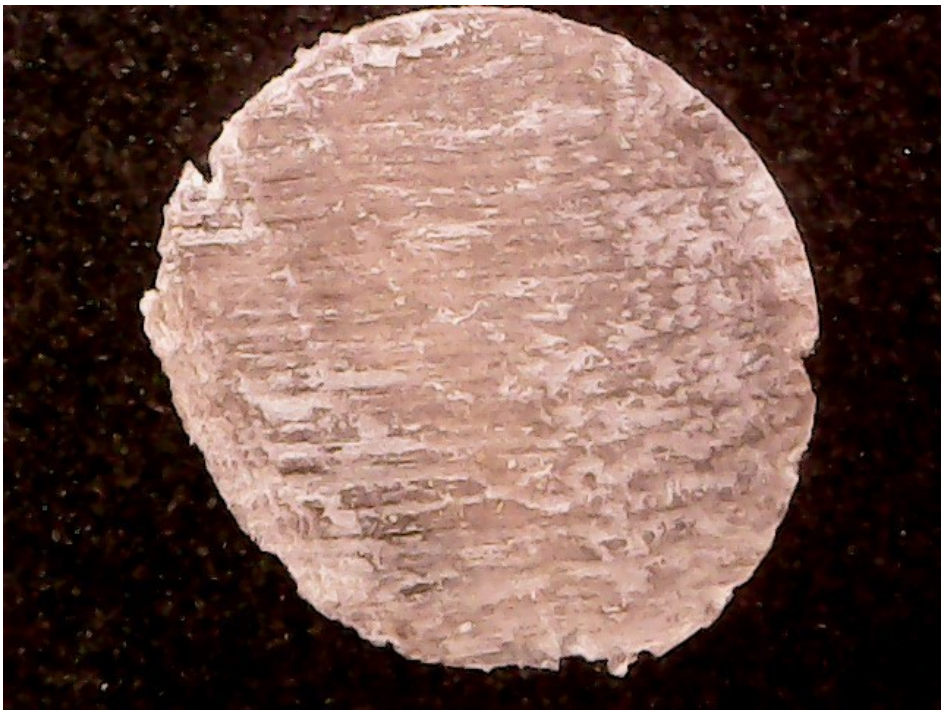

Figure 100. Flaky membrane selected for the experiment

Fungal strains, culture conditions and samples preparation

The strains used in this study were Candida albicans ATCC 1023 and Trichophyton rubrum ATCC 28188. C. albicans was grown at 37°C for 24 hours in Iso-Sensitest Broth (ISB, Oxoid, Basingstoke, UK), and its dilutions were plated in Sabouraud Dextrose Agar (SDA, Oxoid, Basingstoke, UK). T. rubrum was growth at 21-24 °C for 6 days in Potato Dextrose Broth (PDB, Oxoid, Basingstoke, UK) and its dilutions were plated in Potato Dextrose Agar (PDA, Oxoid, Basingstoke, UK).

To evaluate the possible anti-adheisve activity, the membranes were previously immersed for 15 min in benzalkonium chloride 0.4 % in order to eliminate possible contaminations. The membranes were then immersed in 1 ml of sterile water, which was then diluted and plated in TSA, PDA and SDA. No bacterial or fungal growth has been shown.

Phase 1: preliminary test

A preliminary test has been performed on control membranes to assess their capability to be contaminated with the chosen microorganisms. For this purpose, at least 6 membranes have been used. The membrane sections were then soaked in the fungal solution overnight: for both pathogenic agents one section for each contact time (15 minutes, 5 hours, 24 hours) has been used.

At the end of each contact time the membranes were extracted, they were shaken and placed in a Petri dish to dry for a maximum of 20 minutes at 37 ° C. Afterwards, the membranes were put into 2ml of sterile water and from that solution three serial dilutions were made (1: 100, 1: 10000, 1: 1000000). Then all three dilutions were plated using SDA for *C. albicans* and PDA for *T. rubrum,* and then incubated, in aerobic conditions, for 24h at 35-36 °C (*C. albicans*) and for 72h at 21-26 °C (*T. rubrum*). The microbial count has been performed in order to assess membranes contamination.

The morphological characterization of the contaminated membranes was performed with a high-resolution scanning electron microscope (TESCAN, Mira 3 XMU, Brno, Czech Republic).

The discs were directly mounted on aluminium pin stubs, by means of a graphite tape, and coated with carbon using a Cressington 208C (Cressington

Scientific Instrument Ltd, Watford, GB) prior to observation. SEM analysis was performed operating at 20 kV.

Phase 2: In vitro evaluation of a positive and a negative control in reducing fungal adhesion to nail plate

An evaluation of the method was then made using a negative control, a water-repellent film, and a positive control, a commercially available antifungal: Onilaq. Onilaq (Galderma) is a 5% solution of Amorolfine, an active ingredient which is an antifungal belonging to the class of morpholines. It is a well-known drug used for the treatment and prevention of dermatomycosis and onychomycosis [98]. For this experiment the membranes were cut in sections and divided in 5 treatment groups: (1) antifungal treatment (Amorolfine 5% solution, Onilaq, Galderma); (2) water- repellent nail polish; (3) control group; (4) antifungal treatment single application – no wash; (5) water-repellent treatment single application – no wash.

The membranes in groups (1) and (2) were then subjected to three cycles of treatment (at least 18 hours of persistence of the product on the membranes) and washing with a mixture of warm water and hand soap to mimic a normal-life situation of handwashing. The control group (3) underwent three cycles of washing procedure only, while membranes in groups (4) and (5) were treated once without washing before microbiological testing.

Three membranes with the same morphological properties were characterized using Contact Angle Meter DMe-211Plus (KYOWA) before and after water repellent treatment application. The contact angle is a measure of the wettability of a solid by a liquid, in this case treated membranes and water [81]. In general, this assay is used to get information on how the membrane surface interacts with a selected liquid, where high angle values are significant of a poor interaction between the surface and the liquid and a low value indicates high affinity between them. In this specific situation, it has been used to test the efficacy of the water repellent treatment.

For the microbiological assessment, the fungal cultures were prepared in Potato Dextrose Broth for each type of fungal strains with different time of incubation (24 hours for *C. albicans* and 72 hours for *T. rubrum*). Microbial suspensions had an initial inoculum of approximately 2-3x10^6 CFU/ml (colony

forming unit/ml) for C. albicans and 2-3x10^5 CFU/ml for T. rubrum. The treatment groups, (1), (2), (3), (4) and (5), were introduced within the cultures, one for each contact time (15 minutes, 5 hours, 24 hours) for both fungal strains. At the end of each contact time the membranes were extracted, they were shaken and placed in a Petri dish to dry for a maximum of 20 minutes at 37 °C. Then membranes were put into 2ml of sterile water, gently mixed and from that solution three dilutions were made (1: 100, 1: 10000, 1: 1000000). The fungal dilutions were collected on SDA and PDA for the different strains; the plates were incubated, in aerobic conditions, for 24h at 35-36 °C (*C. albicans*) and for 72h at 21-26 °C (*T. rubrum*). After the exposure time for both species the anti-adhesive effect (AE) of the product has been calculated in order to have the logarithmic reduction rate:

AE= log (Nc) – log (Nd)

In which Nc is the colony forming units (CFU) of the control (3) membrane suspension, Nd is the colony forming units (CFU) of the treated sample (after application of (1), (2), (4), (5)) suspension. For each microorganism, 3 replicates have been analyzed for each test. Sterility and growth controls were also included for each time and fungal strain. Results from all membranes over time is reported, expressed as a mean (±SD) of the treatment group. This test was carried out considering AOAC (American Association of Official Analytical Chemists) method for carrier test [99] and CEN method EN13697, with some modifications [100].

Results and Discussion

Phase 1: preliminary test

As reported in figures below the membranes have been contaminated by the two fungal species. The contamination, in the case of T. rubrum is proportional to the contact time (Figure 101), while C. albicans has a peak of growth in the 5 hours and then it showed a modest decline in the 24 hours (Figure 102). That could be explained by the fact that the optimal growth-period of C. albicans is 48 hours and after prolonged exposure the fungal cells are stressed.

Therefore, considering the initial growth-period of the culture (48 hours) and the subsequent contact time with the membranes (24 hours), for a total of 72 hours, a decline in the microbial content after the second exposure time could be coherent.

The fact that there is less growth for T. rubrum depends on the nature of the fungus itself, candida is a yeast and has a faster duplication time, and therefore first colonizes the surface. T. rubrum is a multicellular microorganism, so it has a longer reproduction time and therefore a slower colonization time. The different values depend on the microbial title of the starting culture, as can be seen from the control values.

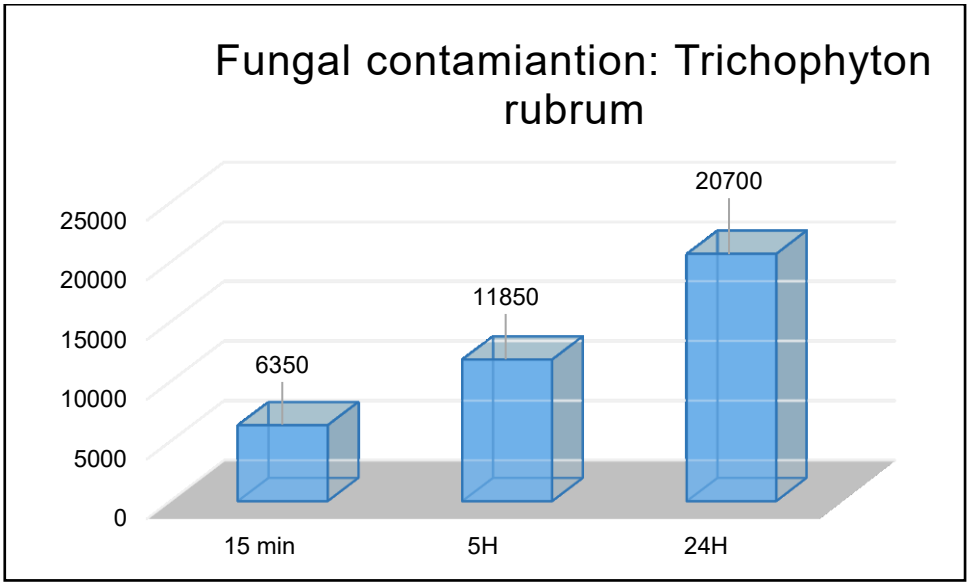

Figure 101. Fungal contamination of T. rubrum (CFU/ml) as a function of the contact time

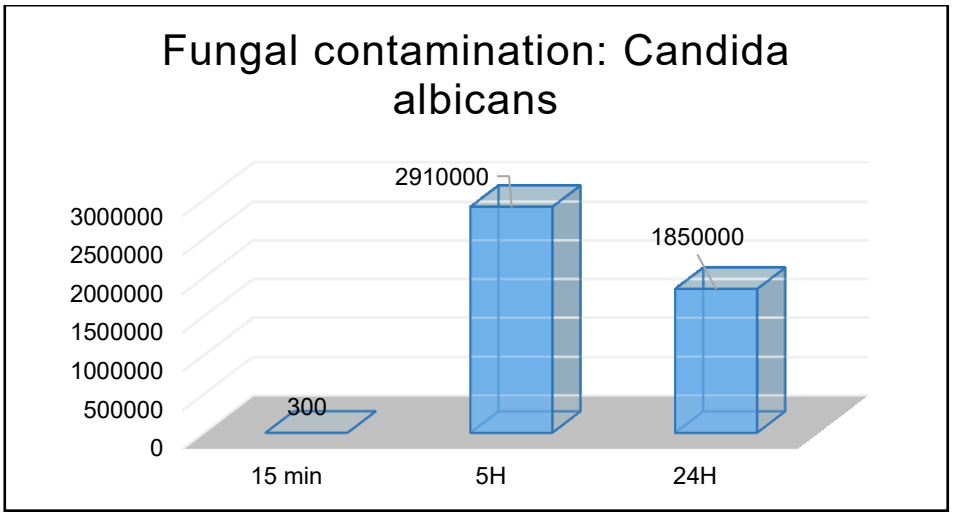

Figure 102. Fungal contamination of C. albicans (CFU/ml) as a function of the contact time

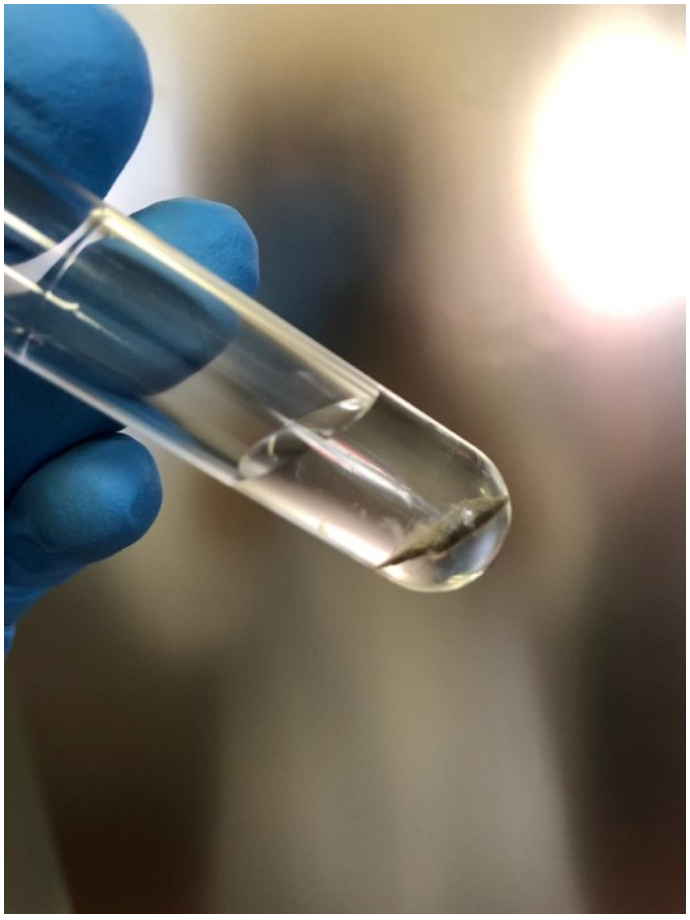

Figure 103. Nail after 24h of contamination with T. rubrum

The SEM analysis confirmed the successful contamination, as it is shown in Figures 104-105.

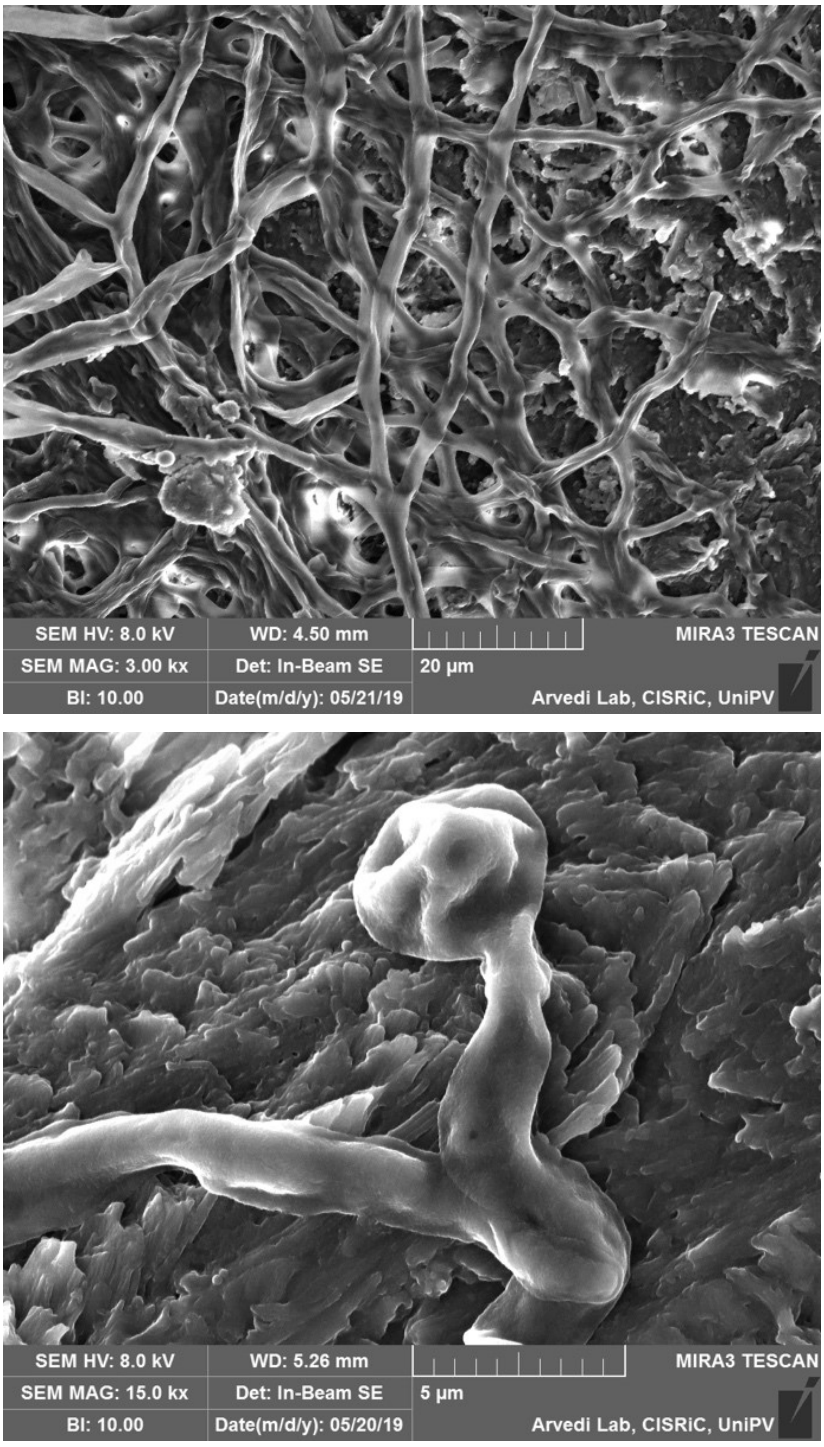

Figure 104. SEM images of a membrane with T. rubrum contamination

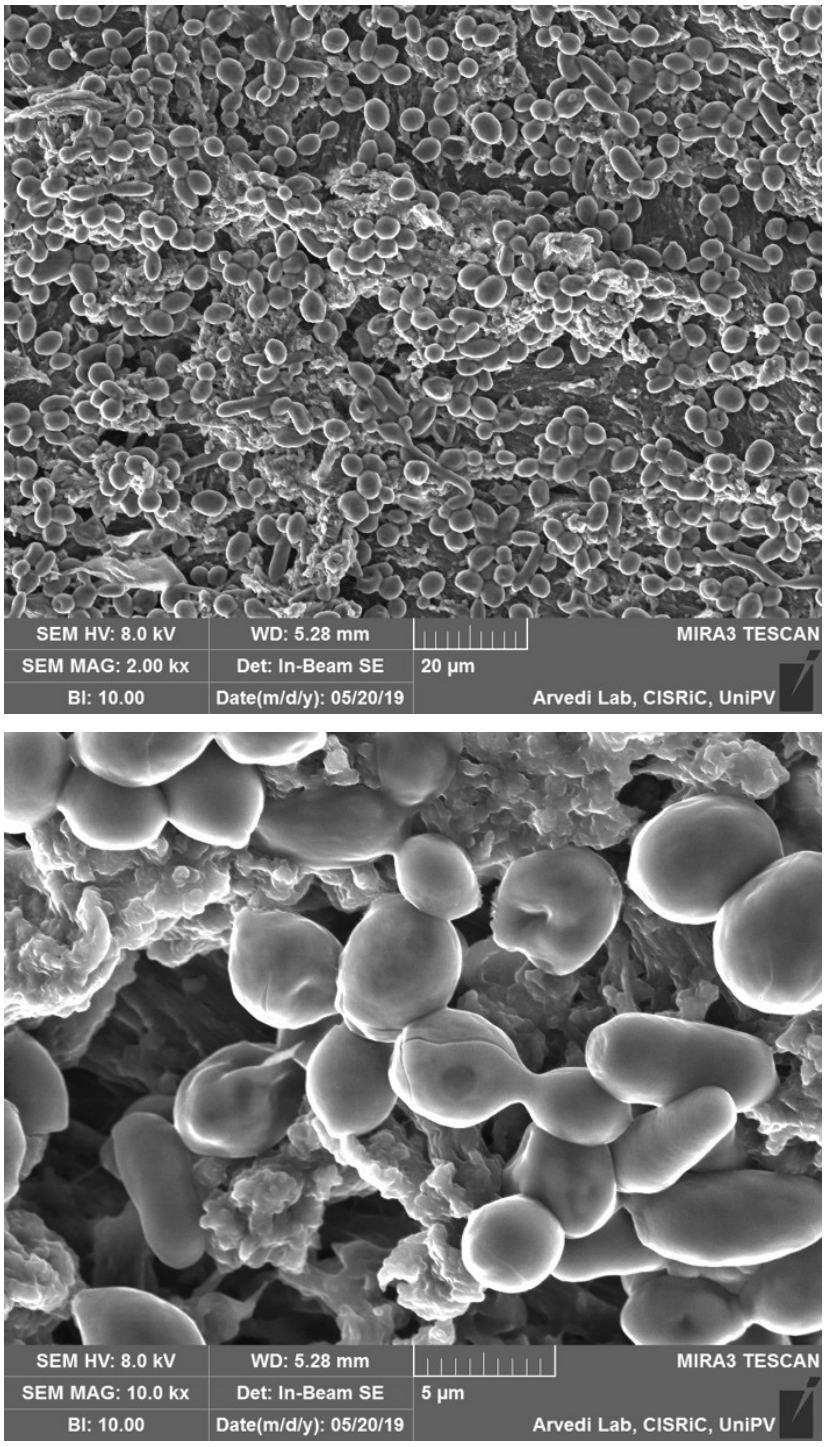

Figure 105. SEM images of membranes with C. albicans contamination

Phase 2: In vitro evaluation of a positive and a negative control in reducing fungal adhesion to nail plate.

In the table below are reported the results of the contact angle measures before (PRE) and after (POST) treatment at three different time points (0, 30

and 60 seconds) after the water drop deposition. The results are expressed as the angle(°) between the surface and the drop.

Table 78. Contact angle measurement after water repellent treatment.

SAMPLE	T (S)	PRE(°)	POST(°)
1	0	87,8	62,3
	30	71,8	62,1
	60	66	59,7
2	0	67,6	75,6
	30	50,7	71,8
	60	42,7	71,1
3	0	65,2	59,6
	30	35,8	58,5
	60	27,1	56,5

Looking at the data reported in table 78. it is possible to appreciate the effect of the water repellent barrier on the membranes. While for the membranes alone there is a reduction in the angle values over time, significant of the high affinity of the liquid for the surface, for the treated membranes the angle is lower in value, which is coherent with the surface more smooth and slippery after the treatment, but constant over time, with almost no variations from 0 to 60 seconds.

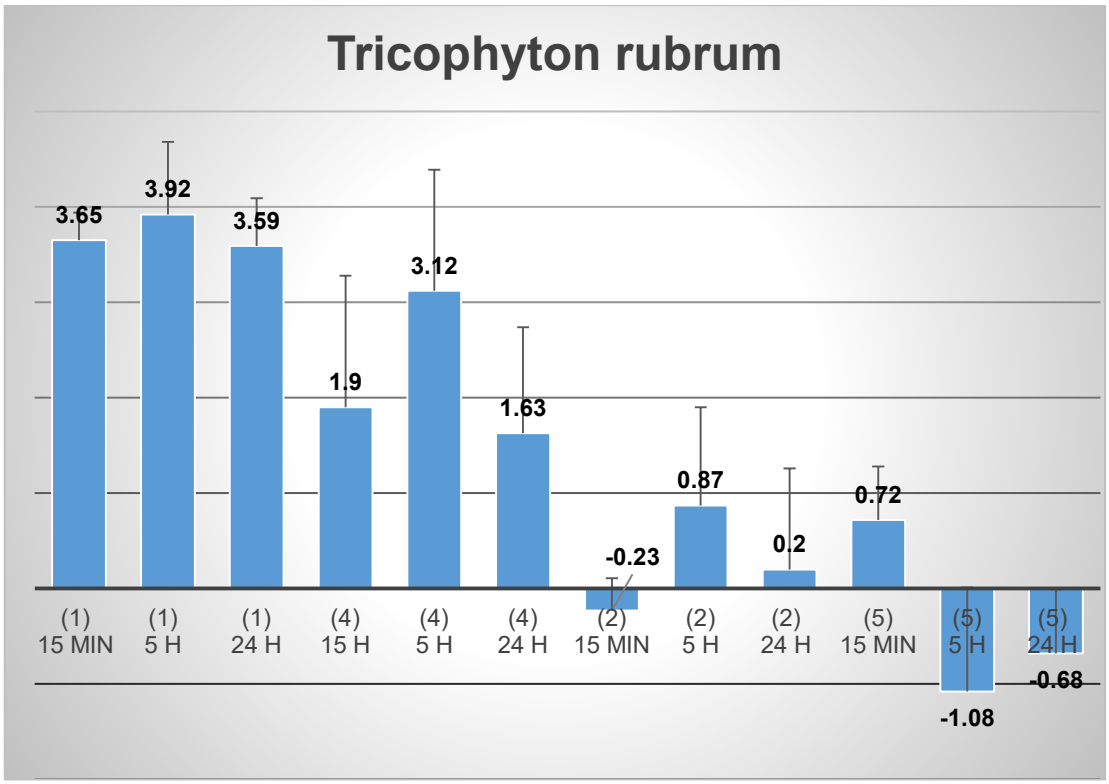

Figure 106. Antiadhesive effect against T. rubrum of (1) antifungal treatment (Onilaq, Galderma); (2) water- repellent treatment (OPI, "start to finish") – (4) antifungal treatment – no wash ; (5) water-repellent treatment – no wash.

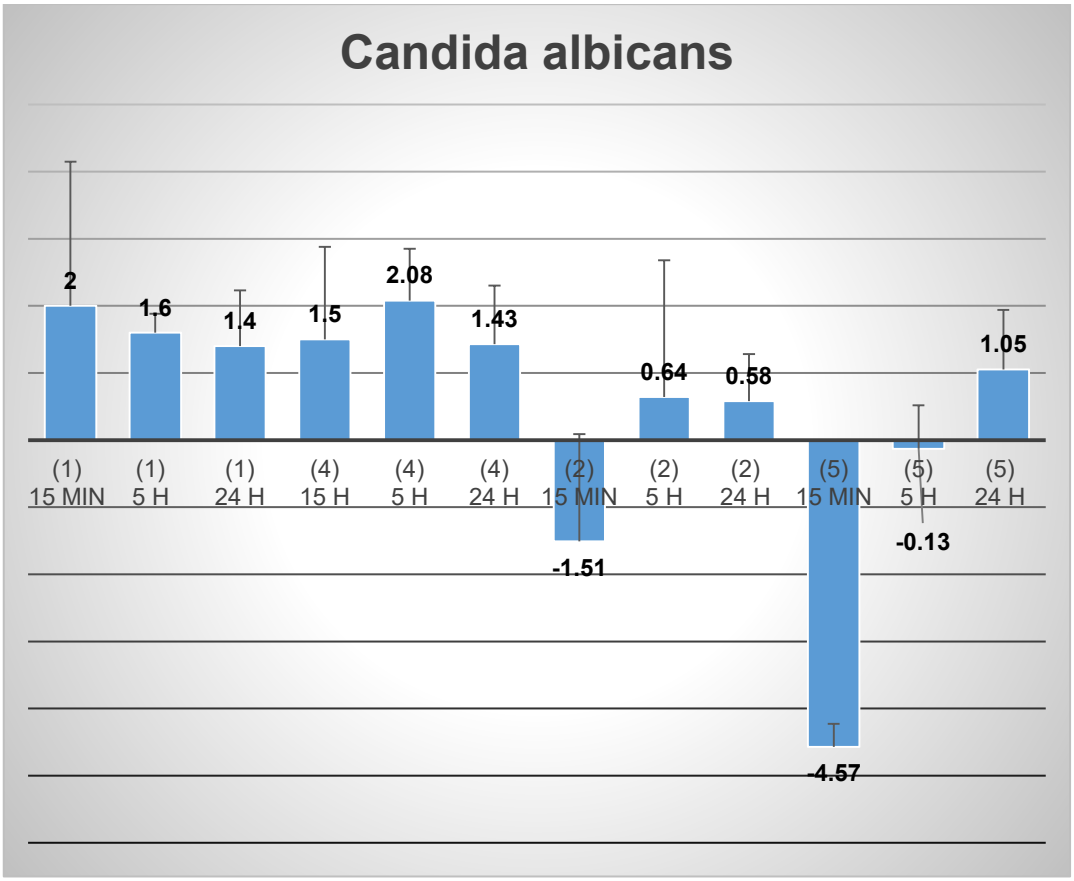

Figure 107. Antiadhesive effect against C.albicans of (1) antifungal treatment (Onilaq, Galderma); (2) water- repellent treatment (OPI, "start to finish") – (4) antifungal treatment – no wash ; (5) water-repellent treatment – no wash.

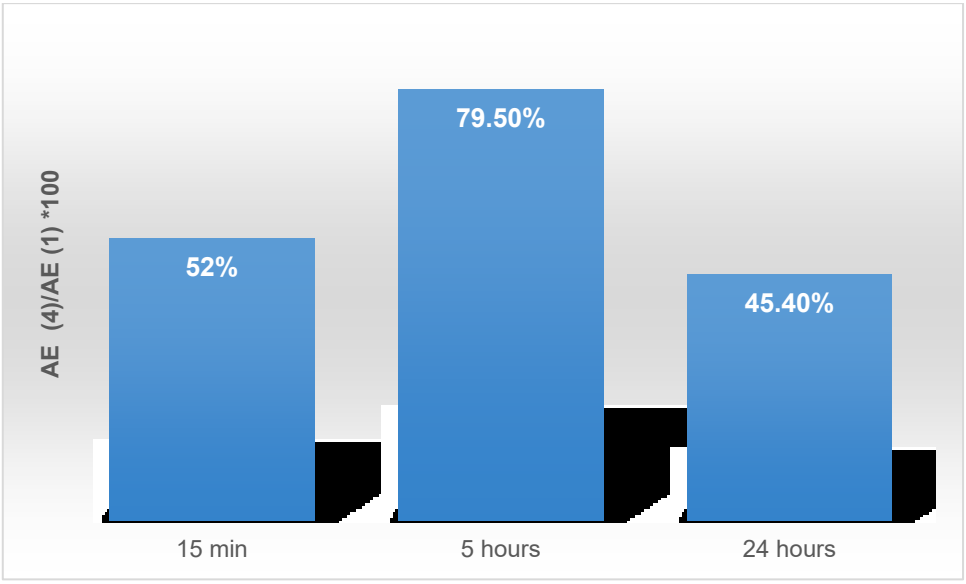

Figure 108. Percentage of the single application anti-adhesive activity compared to the multi-treatment.

The results shown in Figures 106-107-108 report the anti-adhesive effect (AE) of the positive control (antifungal Onilaq) and the negative control (water-repellent film).

For both types of controls, two different application methods were used, as described above:

- Single application of the product (groups (4) and (5));
- Repeated applications of the product and washing procedure (groups (1) and (2)).

As it is shown in the graphs, the performance of the AE seems to be a function of the contact time, presenting the peak of greatest action after 5 hours.

Moreover, the anti-adhesive activity is greater against *Trichophyton rubrum*: in fact, the membranes with antifungal unwashed after 5h had an AE against *T. rubrum* of 3.92 +/- 0.76 and against *C. albicans* of 1.6 +/- 0.28; the antifungal membranes with prolonged treatment and washing had an AE against T. rubrum of 3.12 +/- 1.27 and against *C. albicans* of 2.08 +/- 0.77. This result is coherent with literature showing a higher efficacy shown by Amorolfine against *T. Rubrum* in comparison to the MIC needed for *C. Albicans* [101] [102].

The water-repellent polish instead, has shown consistent results as a negative control, since the AE is much lower and even negative.

After 5h the membranes treated only once shows AE against *T. rubrum* of 0.87 +/- 1.03, and against *C. albicans* of 0.64 +/- 2.04; while the membranes with prolonged treatment and washing show an AE against *T. rubrum* of -1.08 +/- 1.09 and against *C. albicans* of -0.13 +/- 0.65.

What it is possible to assume from these results is also the fact that prolonged application with washing procedure has a stronger impact on the AD effect of the product, protecting more the membranes from a fungal attack than a single application did. Moreover, even for the negative control after several washes the results are more significant, with no AD effect shown.

Conclusion

The applied protocol for membranes contamination has shown to be effective (Phase 1) for the fungal species used. Thus, hoof membranes have shown to be a valuable in vitro model to consider for this kind of product assessment. However, some challenges have been faced with the model and they need to

be taken into account, such as the flaking of the membrane itself which becomes more permeable in an aqueous environment.

Phase 2 experiment, in fact, has shown that the most significant AE value is obtained after 5 hours: 15 minutes is an insufficient time for the fungi to establish permanent contact with the membranes, both treated and untreated, while 24h is probably too long for the nail to remain in contact with a very high fungal load in an aqueous solution.

Even 5 hours in this sense could be a very long time, however the results obtained from the positive control allow us to understand how active an antifungal already tested and on the market is.

Results obtained in Phase 2 experiment showed that one single application is not enough for the product to be effective, and the membrane must instead be treated several times with the product under testing to show significant activity.

CONCLUSION

The aim of this three years' work has been the development of protocols ideally suitable to become the standard procedure to apply in order to assess safety and efficacy of nail products in vitro, in both the cosmetic and pharmaceutical field.

The main goal of the first part of the work was to evaluate and test a complete range of characterization techniques on hoof membranes as nail in vitro model. As stated above, the production method developed by our research group has shown to be effective, making it possible to obtain membranes with of desired thickness, homogenous within thickness group. Moreover, the feasibility assessment of membranes characterization process through several surface techniques has been mainly successful.

In particular, mechanical properties analysis applying the Nail StrainStress meter NM100 has proved to be challenging but efficient, showing the full potential of the hoof membranes as a fruitful model to simulate unhealthy nails. Mechanical properties of nail are maybe their most distinguished biophysical feature, and among the first ones affected when a disease condition arises. Nail pathologies often have a strong impact on mechanical properties of the lamina: the nail plate becomes more fragile as a primary symptom of the disease, such as for brittle nail syndrome, but also as a secondary effect of other wide diffuse nail pathologies like onychomycoses. Moreover, nails pathological manifestations can be the first alarm of systemic disease.

For these reasons, mechanical properties have been chosen as the main focus of the cosmetics application of the model. In fact, the protocols reported in chapter 2 were developed to assess cosmetics products designed to address fragile nails conditions. From the results obtained, it can be stated that the in vitro studies in question objectively sustained most of the commercial claims of those products and on a more general level bovine hooves membranes demonstrated their suitability as in vitro model to assess efficacy in this field.

The intrinsic variability of the model properties has been controlled in a satisfactory way with strict selection criteria for the suitable membranes to be used for the studies. Since hoof membranes have demonstrated to be suitable to be characterized with a wide range of techniques, the addition of other inclusion and/or exclusion criteria within the selection process of membranes could be a solution in terms of variability reduction, at the same time however, it would be challenging in terms of time and number of samples required.

This work has laid the foundations for the analysis of the correlation among the different hoof membranes properties analysed, both mechanical and surface related. A deeper understanding of the relationships among the different characteristics and the 'origin' of the membranes, if standardized and validated, could considerably reduce the time needed for the selection process.

Hydration is a critical parameter to be considered when dealing with this kind of samples and consequently in the process of protocol development and products evaluation. It is well-known to have a strong impact on nails, since the water-content of the lamina can possibly induce modifications in permeability and mechanical properties. Water content and TOWL could be other two parameters to be considered both when selecting membranes at the beginning and as an endpoint of the study.

As stated above, this research offers not only a tool to objectively support products commercial claims, but also a way to assess products safety through a model which resembles a real-life situation as close as possible.

Nail products assessment suffers of a lack of standardization also in the pharmaceutical field.

Onychomycoses, despite its high incidence worldwide, it is still hard to be successfully treated. This pathology has been the focus of the third chapter of this work, centred on the development of in vitro protocols to address onychomycoses through the use of novel techniques. Three different projects have been developed to cover different stages of the disease: in particular one attends to prevention and two which examines new treatments assessment.

Concerning prevention, a feasibility study on a novel protocol to evaluate anti-adhesion efficacy has been proposed.

With regard to anti-fungal treatments, an in vitro efficacy assessment of drugs alone and in combination against *T. rubrum* and a permeability in vitro study of a product through the use of an innovative apparatus have been carried out successfully.
Despite the difficulties already highlighted in the relevant sections, each project has shown promising results both in terms of the potential of the in-vitro protocols developed to be applied for treatment efficacy evaluation and the suitability of hoof membranes model to be used for a wide range of in-vitro test, far beyond permeability studies applications.

It is rather complex to bring forward changes and promote standardization in a very specific and relatively fringe research field like this is.
However, a standardized in-vitro assessment procedure could be key for future developments. Cosmetics products are mostly assessed through in vivo studies, which could be very time consuming and to some extent biased, while pharmaceutical pre-clinical research is rapidly evolving, guided by the ethical spirit of the three Rs: replacement, reduction and refinement. It is then of critical importance to develop reliable assays for treatment efficacy and safety assessment in those fields where they are still missing.

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