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Faculty of Tropical AgriSciences

Effect of saponin-rich extracts from *Quillaja saponaria* solely and in combination with *Yucca schidigera* on adhesion of lactobacilli

MASTER'S THESIS

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Declaration

I hereby declare that I have done this thesis entitled Effect of saponin-rich extracts from *Quillaja saponaria* solely and in combination with *Yucca schidigera* on adhesion of lactobacilli independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

In Prague, 25th April 2024

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Eva Vacíková

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Abstract

Quilaja saponaria, a tree native to Chile, and *Yucca schidigera*, a plant native to the deserts of North America, are important for their content of secondary metabolites with an excellent biological activity and proven safety. Saponins from these two species are used as ingredients in food, beverages, cosmetics as a medicine and as additive for livestock feed. It has been demonstrated that these saponins are beneficial in lowering ammonia emissions, litter moisture, and footpad dermatitis in chickens. They can also improve food digestibility and thus promote growth, provide antioxidant activities, reduce coccidiosis-related damages, etc. Due to these properties, these saponins have great potential in replacing antibiotics used commercially in animal fattening. The aim of this thesis was to determine the cytotoxicity of the pure *Q. saponaria* extract (QS) and extract of the combination of *Q. saponaria* and *Y. schidigera* (QY) and then to test the adhesion of probiotic lactobacilli in the presence of these extracts to human carcinoma colonic epithelial cell line. The plant extracts were digested using the static *in vitro* static digestion model INFOGEST and subsequently used in cytotoxic and adhesion experiments on the cell culture model. For the determination of cytotoxicity, the MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium Bromide) cytotoxicity assay was used to measure the cell viability; 3 common human lactobacilli (*Lactobacillus brevis, L. gasseri and Lacticaseibacillus rhamnosus*) and co-culture of epithelial cell lines (Caco-2 and HT29) was then used in the adhesion experiment. The cytotoxicity of the extracts was determined separately on two cell lines HT29 and Caco-2. The cytotoxicity of QS extract was IC_{20} = 172 and IC_{50} = 419 μg/ml for the Caco-2 cell line, while no cytotoxic effect was observed for the HT29 cell line. In the case of QY extract IC_{50} = 304 μg/ml for the Caco-2 cell line. No IC_{20} was determined for Caco-2 cell line as well as no cytotoxic effect was observed for the HT29 cell line in tested concentrations of QY extract. Adhesion results showed that QS and QY extracts significantly reduced the adhesion of all bacteria species tested. The only exception was *L. rhamnosus*, for which the QY extract caused an increase in adhesion at the lowest concentration. In general, QY extract reduced adhesion much less than QS extract. Only in *L. rhamnosus* was the decrease in adhesion clearly dependent on the concentration of both extracts used.

Key words: saponins, adhesion, Soapbark, Mojave yucca, cytotoxicity

Abstrakt

Quilaja saponaria, strom pocházející z Chile, a *Yucca schidigera*, rostlina pocházející z pouští Severní Ameriky, jsou důležité pro svou vynikající biologickou aktivitu a prokázanou bezpečnost. Saponiny z těchto dvou druhů se používají jako přísady do potravin, nápojů, kosmetiky, jako lék a jako přísada do krmiv pro hospodářská zvířata. Bylo prokázáno, že tyto saponiny mají příznivý vliv na snížení emisí amoniaku, vlhkosti podestýlky a dermatitidy paznehtů u kuřat. Mohou také zlepšovat stravitelnost krmiva, a tím podporovat růst, poskytovat antioxidační účinky, snižovat škody způsobené kokcidiózou atd. Vzhledem k těmto vlastnostem mají tyto saponiny velký potenciál nahradit antibiotika, která se komerčně používají ve výkrmu zvířat. Cílem této práce bylo stanovit cytotoxicitu čistého extraktu z *Q. Saponaria* (QS) a extraktu z *Q. saponaria* a *Y. schidigera* (QY) v kombinaci a následně otestovat adhezi probiotických laktobacilů v přítomnosti těchto extraktů na epiteliální buněčné linii střevního karcinomu. Rostlinné extrakty byly ztráveny pomocí statického *in vitro* modelu trávení INFOGEST a následně použity v cytotoxickém a adhezním experimentu. Pro stanovení cytotoxicity byl použit test cytotoxicity MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-difenyltetrazolium bromid) pro měření životaschopnosti buněk; 3 běžné lidské druhy laktobacilů (*Lactobacillus brevis, L. gasseri* a *Lacticaseibacillus rhamnosus*) a ko-kultura epiteliálních buněčných linií (Caco-2 a HT29) pak byly použity v adhezním experimentu. Cytotoxicita extraktů byla stanovena zvlášť na dvou buněčných liniích HT29 a Caco-2. V případě QS extraktu vyšla cytotoxicita vůči Caco-2 buňkám následovně IC_{20} = 172 a IC_{50} = 419 μg/ml. U buněčné linie HT29 nebyl pozorován žádný cytotoxický účinek QS extraktu. V případě extraktu QY byla cytotoxicita vůči Caco-2 buňkám IC_{50} = 304 μg/ml. U buněčné linie CaCo-2 nebyl u QY extraktu stanoven cytotoxický účinek na úrovni IC_{20} a stejně tomu tak bylo i u buněčné linie HT29. Výsledky adheze ukázaly, že extrakty QS a QY významně snížily adhezi všech testovaných druhů bakterií ve většině koncentrací. Jedinou výjimkou byl *L. rhamnosus*, u kterého extrakt QY způsobil zvýšení adheze při nejnižší koncentraci. Obecně lze říci, že extrakt QY snižoval adhezi méně než extrakt QS. Jen u *L. rhamnosus* bylo jednoznačně snížení adheze závislé na použité koncentraci obou extraktů.

Klíčová slova: saponiny, adheze, Mýdlokor, Mojave juka, cytotoxicita

Contents

List of tables

List of figures

List of the abbreviations used in the thesis

AGP – Antibiotic growth promoters

BW – Body weight

EMEM – Eagle's minimum essential medium

FDA – Food and Drug Administration

GIT – Gastrointestinal tract

GRAS – Generally recognized as safe

LAC – Lactobacilli

MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

MUC – Mucin

QS – *Quillaja saponaria*

QY – *Quillaja saponaria* in combination with *Yucca schidigera*

RP-HPLC – Reversed-phase high-performance liquid chromatography

SGF – Simulated gastric fluid

SIF – Simulated intestinal fluid

SSF – Simulated salivary fluid

WHO – World Health Organization

YS – *Yucca schidigera*

1. Introduction

Quillaja saponaria a tree growing in Chile and *Yucca schidigera* a plant growing in the southwest deserts of North America are important sources of saponins, secondary metabolites, with a wide range of applications. These saponins are well known for their cytotoxic, antiviral, and immunological properties. Saponins have also found great use as additives in livestock feed. It has been shown that these saponins can reduce atmosphere ammonia, litter wetness, and footpad dermatitis in chickens. *Quillaja* and *Yucca* saponins can also improve food digestion, hence promoting growth, provide antioxidant activity, and mitigate coccidiosis-related harm. Because of these properties, saponins have a high potential for substituting commercially available antibiotics in animal fattening. The aim of this study was to determine the cytotoxicity of *Quillaja saponaria* extract and extract of the combination of *Quillaja saponaria* and *Yucca schidigera* and subsequently evaluate how these extracts affects the adhesion of selected probiotic microorganisms of the genus *Lactobacillus* to the intestinal epithelium.

2. Literature Review

2.1. Quillaja saponaria

Molina (1782) established the genus *Quillaja*, with *Quillaja saponaria* (QS) as the type species. QS was the only one recognized species belongs to this genus until a century ago. Originally was QS categorized in the Rosaceae family, but molecular and morphological phylogenetic research validated its placement in the Quillajaceae D. Don, order Fabales Bromhead, and superorder Rosanae Takhtajan (Bello et al. 2012; "Tropicos" n.d.).

The species *Quillaja* includes *Quillaja saponaria* Mol. and *Quillaja brasiliensis* (A. St.-Hil. & Tul.) Mart. (Luebert 2013). The popular common name for *Quillaja* species in Spanish is "quillay", or "küllay" in the Mapuche language. It has also been referred to as "jabón de palo" and "palo jabón" (tree soap and soap-tree) in the Spanish language (Muñoz-Schick et al. 2012). *Quillaja* is known as soapbark or Panama bark in English, "Seifenrinde" and "Panamarinde" in German, and "bois de Panama" in French, referring to its export via Panama from the Spanish colonial era (Delporte et al. 2021).

Synonyms: *Quillaja poeppigii* Walp., *Quillaja molinae* DC., *Smegmaria emarginata* (Ruiz & Pav.) Willd., *Smegmadermos emar- ginatus* Ruiz & Pav. (Rodriguez et al. 2018).

2.1.1. Morphological description

QS is the evergreen tree that has a trunk diameter of up to one meter and can reach heights of over twenty meters. Its bright green hairless leaves are coriaceous, simple, alternate, oblong, and short-petioled, either smooth-edged or slightly dentate.

Its hermaphroditic, flattened, star-shaped flowers (Figure 1) are either single or grouped in corymbose racemes that arise from the leaf axils. The calyx has five sepals, while the corolla has five petals. The androecium is composed of ten stamens that are organized in two series of free filaments. The pistil is made up of five ovaries, each with its own filiform style and stigma. Once the fruits mature, the five carpels that are initially connected to the base give rise to five dehiscent woody follicles grouped in

a star arrangement, each bearing several winged seeds (Hoffmann et al. 1992; Montenegro 2000). When a tree is young, its bark is smooth and light brown (Figure 2), but as it ages, it takes on a deeper greyish tint with longitudinal fissures. Under a microscope, it displays large sclereids, hexagon-shaped calcium oxalate crystals, lignified fibres, pieces of brown amorphous masses, spherical starch granules, and sparse suberized cells (Cañigueral et al. 1998).

Figure 1. *Quillaja saponaria* – leaves, flowers. Source: Delporte et al. (2021)

Figure 2. *Quillaja saponaria* – trunk. Source: Delporte et al. (2021)

2.1.2. Geographical distribution

QS is one of the most significant tree species of the Chilean evergreen sclerophyllous forest type, according to Benedetti et al. (2000). It grows in the transition zone between the southern deciduous forests and the semiarid Ovalle (IV Region of Coquimbo) and Collipulli (IX Region of Araucanía) in Central Chile, between 30°30′ and 38° S (Figure 3).

The wide range of environmental conditions in which this species thrives, from the western part of the coastal zone to the northern and southern foothills of the Andes, should be highlighted (Gotor 2008, in Correa V. & Martínez C. 2013). The minimum and maximum mean temperatures range from −3.2 °C to 9.4 °C and 16.5 °C to 31.3 °C (Correa V. & Martínez C. 2013) respectively, with extremes of −5.7 °C and 40.7 °C The Quillaja forests are spread over approximately 320,000 hectares and their biomass yield is estimated at 4.5 tonnes per 0.5 hectare (Magedans et al. 2019).

Figure 3. Ditribution of *Quillaja saponaria*. Source: MapChart, Author (2024)

2.1.3. Ecological requirements

The Mediterranean climate, with average annual temperatures of around 14 °C and winter rainfall between 150 and 1500 mm, is ideal for the growth of QS (Correa V. & Martínez C. 2013). It grows mostly in sunny, flat regions and hillsides that are up to 1500 meters above sea level, frequently in areas with little vegetation (Hoffmann et al. 1992; Gupta 1995). QS is able to effectively absorb nutrients and water from the soil due to the depth and horizontal development of its root system. This trait has led to the species' employment in soil stabilization, afforestation, and the rehabilitation of degraded land because of its great ability to grow in eroded and poor soils (Valenzuela & Lizzie 2007; Correa V. & Martínez C. 2013). QS often creates communities with other hard-leaved species including "boldo" (*Peumus boldus*), "maitén" *(Maytenus boaria*), "peumo" (*Cryptocarya alba*), "litre" (*Lithraea caus-tica*), and "huingán" (*Schinus polygamus*), besides others. It also grows in groups with Nothofagus spp. in Chile's mesomorphic vegetational zone further south (Vita 1974; Novoa Cortez 2010; Correa V. & Martínez C. 2013). Shade-tolerant plants such as *P. boldus* and *C. alba* displace this intolerant species under higher humidity. Research was conducted to determine how much water and fertilizer *Quillaja* needs, specifically how much

nitrogen, phosphorus, potassium and boron should be applied to achieve optimum development of this tree (Delporte et al. 2021). According to the findings of Valenzuela & Lizzie (2007), irrigation was the main factor that promoted the survival of QS. Fertilizer treatment plus irrigation resulted in a higher percentage of survival of young plants in the field. Overharvesting has led to the declaration of this species as vulnerable. Consequently, in order to harvest it or use it for any other purpose, permission from the Chilean Agriculture and Livestock Service (abbreviated SAG) is required. This agency only issues permissions for individual trees that do not form a forest (Delporte et al. 2021).

2.1.4. Traditional use

Bark and leaf infusions of QS were used in ethnomedicine to treat skin disorders and respiratory ailments (as an expectorant). The injection given vaginally was intended to prevent leukorrhea and bleeding. Furthermore, "quillay" bark is still thought to promote stomach secretion, function as a diuretic, and cure baldness. Its blossoms have been used in baths to treat chronic rheumatism and as an infusion to massage limbs and afflicted regions, while the Mapuche apply the bark to treat toothache and as a cleaner (Hoffmann et al. 1992; Zin & Weiss 1998). The term "quillay" was first used in Europe by Frézier & Picart (1717), who described that "its bark foams in water, like soap, making it better (than soap) for washing woolen goods but not for linen, which it stains yellow. All Indians use it for washing their hair and cleaning their heads; it is thought that this is what causes their hair to be black." According to Molina (1810), the author of the scientific name of *Quillaja saponaria*, the most precious part for Chileans is its bark, which, when crashed and dissolved in water, creates a foam like the most flawless soap, removes stains effectively, defats wool, and cleans all kinds of fabric and cloth in the best way. As a result, the Peruvians harvest a huge amount of this bark.

2.1.5. Modern medicine

Quillaja saponins have caught the interest of scientists because of their cytotoxic, antiviral, and immunological adjuvant properties (De Sousa et al. 2004; Roner et al. 2007; Fleck et al. 2019). Saponin-rich "quillay" extracts have also been demonstrated in studies to be larvicidal, fungicidal, hepatoprotective, and nematocidal

(Martín & Briones 1999a; Pelah et al. 2002). The less toxic saponin fractions of *Quillaja* species have been used experimentally in the treatment and prevention of infectious diseases, autoimmune disorders, and cancer, as a treatment for pneumococcal infections and AIDS, in the prevention of malaria, and in the treatment of tuberculosis (Kensil et al. 1991). More important immune response adjuvants are several fractions of QS aqueous bark extracts. Increased *in vivo* antibody titers can be obtained by establishing these connections with certain anti-genes. For instance, QS -21 increased the production of cytotoxic T- lymphocytes, the Th1 cytokines interleukin-2 and interferon-gamma, and IgG2a antibodies (Kensil et al. 1991; Pham et al. 2006). Additionally, clinical research showed that QS-21 much surpassed other adjuvant classes, such as peptidoglycans, bacterial lipopolysaccharides, bacterial nucleosides, and glucan formulations, in boosting T-cell responses and anti-body responses against target antigens (Fleck et al. 2019). QS-21A, which is a synthesised blend of QS-21A_{xylose} and QS-21A_{apiose}, has been shown by Kim et al. (2006) to be one of the most potent and safe immunoadjuvants. QS-21A creates the bonds with antigens found in certain types of cancer, HIV glycoproteins, and malaria protozoan proteins, increasing the immunological response mediated by T and B cells in microgram dosages. Regarding the structural requirements for the immunoadjuvant activity of quillaja saponins, the aldehyde group at C-23 of the aglycone is essential for Th1 promoting activity, presumably because it can form Schiff's bases with the amino groups of some T-cell receptors; while the hydrophobic acyl side chain at C-28 of QS saponins can stimulate the cytotoxic response of CD8+ T-cells and also play an important role in the toxicity of certain saponin fractions (Fleck et al. 2019). Recent research has focused on the anti-inflammatory properties of quillaic acid and its derivatives against phorbol ester (TPA) or arachidonic acid (AA). Quillaic acid had significant topical anti- inflammatory efficacy in both experiments, and several of its compounds outperformed synthetic nonsteroidal anti-inflammatory medications. Based on the structural alterations and biological data, it appears that the anti-inflammatory effect in these models is related to the aldehyde group at C-23 and the carboxyl group at C-28 (Zhang & Popovich 2009; Rodríguez-Díaz et al. 2011). Furthermore, in two murine thermal models, Arrau et al. (2011, 2019) showed that quillaic acid, its methyl ester, and an oxidized derivative of the latter produce dose-dependent antinociceptive effects. These findings were

established *in vivo*. The saponin mixture's results, which were obtained, validate the traditional use of this species as an analgesic.

In trade, the term "quillaia" refers to the powdered inner bark, leaves, and wood of *Q. saponaria* (and maybe also *Q. brasiliensis*). The term "quillaia extract" refers to the aqueous extract that is registered as a food additive (E 999). This substance can be found as a surfactant in cosmetics and is also utilized as an emulsifier and foaming agent. The Panel on Food Additives and Nutrient Sources Added to Food of the European Food Safety Authority has published a scientific opinion on E 999 as a food additive and assessed the safety of its suggested usage in flavorings (EFSA 2019). Recently, the interfacial effects of the saponins from QS and their complexes with other surfactants were evaluated (Reichert et al. 2019). *Quillaja* bark exports declined from 420.869 kg in 2002 to 159.837 kg in 2018 as a result of increasing shipping of spray-dried aqueous extracts, which jumped from 182.302 kg to 618.491 kg at the same time, making up about half of total exports to the United States (Delporte et al. 2021). It is worth noting that, since the initial idea twenty years ago (Martín & Briones 1999a) these products are increasingly produced from all above-ground components of farmed saplings rather than adult trees, which may be destroyed in the process (Desert King International 2019).

2.2. Genus *Yucca*

The genus Yucca contains 50 species. The majority are more xerophytic than succulent. Many *Yucca* species are important in horticulture or as prominent features in arid vegetation.

Yucca is easily distinguished by the flat and thin leaves common to many species, frequently with filiferous leaf margins found only in *Hesperaloe* and a few *Agave* species. The genus is further distinguished by its typically whitish wax-like and rather mushy dangling flowers in usually compact inflorescences. The inflorescences are either racemose or paniculate, and sometimes racemose distally; the part-inflorescences are invariably unbranched and usually shorter distally (Thiede 2020).

2.2.1. *Yucca schidigera*

Yucca schidigera Roezl ex Ortgies (1871), belonging to the Asparagaceae family, is a flowering plant indigenous to the southwest deserts of North America. It is often referred to as the Mojave yucca or Spanish dagger.

Yucca schidigera (YS) is a tiny evergreen (Figure 4) tree with a prominent base trunk and a dense crown of spirally arranged bayonet-like leaves. The gray-brown bark is dotted with brown, dead leaves toward the top, and as it gets closer to the ground, it becomes sporadically rough, scaly, and ridged. The leaves are thick, extremely rigid, concavo-convex, and range in color from yellow-green to blue-green. They measure 30-150 centimetres in length and 4-11 cm in width at the base. The flowers are divided into six sections and are bell-shaped, measuring 3–5 cm in length (sometimes up to 7.5 cm). They are white, occasionally tinged purple, and are produced in a compact, bulbous cluster that is 60–120 cm tall at the top of the stem. The berry is up to 11.5 cm long and elongated (Thiede 2020).

Figure 4. *Yucca schidigera*. Source: Thiede (2020)

2.2.2. Uses of *Yucca schidigera*

Yucca schidigera is a medicinal plant from the North Amerika. According to traditional medicine, YS extracts contain anti-arthritic and anti-inflammatory properties. Native Americans used this herb in traditional medicine to treat a wide range of conditions, including arthritis. YS offers several physiologically active compounds. It contains a high concentration of steroidal saponins and is utilized commercially as a saponin source. YS products are being used in a variety of applications. YS powder and extract are used as animal feed supplements. Beneficial effects in livestock and poultry production include increased growth rate and feed conversion efficiency, lower atmospheric ammonia levels in confinement animal and poultry facilities, anti-protozoal and nematocidal activity, modification of ruminal microbe populations, inhibition of Gram-positive bacteria, lower egg and tissue cholesterol levels, and anti-arthritic activity in horses and dogs. Other applications include using yucca extract as a foaming agent in beverages (Cheeke et al. 2006). Yucca offers a variety of phytochemicals, which are contributing to these effects. Steroidal saponins are the most well-known. Saponins are natural detergents that produce stable foam (Cheeke 1998).

2.3. Saponins

More than 500 plant species contain saponins, whose concentration in plant extracts ranges from 0.1% to 10% (Cheeke 1998). Saponins, biologically active toxic compounds, are produced by plants to protect themselves from pathogens' attacks. Saponins are secondary metabolic compounds produced by healthy plants with potential antipathogenic activity and can serve as a chemical barrier against pathogens. There are two main categories of saponins: steroidal and terpenoid saponins (Zaynab et al. 2021).

Saponins can be obtained from *Quillaja*, *Yucca*, soybean, chickpea, spinach, ginseng, sugar beet, sunflower, oats, and many other plant species. Saponins can be extracted from their bark, seeds, roots, leaves, fruits and stems (Oakenfull 1981; Güçlü-Üstündağ & Mazza 2007). Other sources of saponin have also been found, in marine organisms such as starfish (Oakenfull 1981). The term "saponin" alludes to the interfacial activity seen for this family of compounds and is derived from the Latin word "sapo," which means "soap" (Hostettmann & Marston 1995). Although some saponins have been reported to have a sweet or neutral flavour, saponins are often

sensory active, which means they frequently have a distinctive bitter taste (Güçlü-Üstündağ & Mazza 2007). Saponins are chemically classified according to the hydrophobic backbone, which consists of either a steroid or a sugar-free triterpene aglycone; also referred to as sapogenin. One or more sugar moieties are usually attached to sapogenin, giving the molecule an amphiphilic nature (Hostettmann & Marston 1995).

Hostettmann & Marston (1995) developed a sugar-based classification scheme based on the varying structures of sapogenins and attached sugar moieties. A monodesmosidic saponin has one sugar moiety attached at the C-3 position, whereas bidesmosidic saponins have two attached sugar chains and tridesmosidic saponins have three attached sugar chains.

Sugar moieties of steroid saponin structures are typically attached at the C-3 and C-26 positions in bidesmosidic saponins, whereas sugars are frequently attached at the C- 3 and C-28 positions in triterpenes. The most well-known saponins are bidesmosidic. However, there are a few saponins with more sugar chains attached (Güçlü-Üstündağ & Mazza 2007), such as the recently identified tridesmosidic saponins from the tree *Koelreuteria paniculate* Laxm. (Mostafa et al. 2016). Sugar moieties are typically made up of two to five monosaccharides, such as d-glucose, d-galactose, d-glucuronic acid, d-galacturonic acid, l-rhamnose, l-arabinose, d-xylose, and d-fucose (Hostettmann & Marston 1995).

2.3.1. Saponins of *Quillaja saponaria*

The evergreen QS grows only in certain areas. According to Schlotterbeck and co-authors (Schlotterbeck et al. 2015), native QS trees grow only between 30°S and 38°S due to specific climatic requirements (Donoso et al. 2011).

QS is derived from the Chilean word quillean, which means "to wash," and is related to the washing activity of QS saponins. Because of their wide applicability in the food, cosmetic, and pharmaceutical industries, the demand for QS saponins for industrial use has increased significantly since then. There are currently only a few commercially available saponin products that are almost entirely derived from the QS tree (Dalsgaard 1978). The approximate amount of biomass needed for industrial applications from QS trees by 2019-2020 ranges from 40,000 to 48,000 tons

(Schlotterbeck et al. 2015). Because of the high demand, researchers are working to find alternative sources or develop formulations that use fewer QS saponins as active ingredients. Originally, only the bark of QS trees was used as a saponin source, resulting in QS bark overexploitation (Rundel & Weisser 1975). This resulted in a significant decrease in the number of naturally growing QS trees. The Chilean National Forestry, therefore, enacted legislation to limit the exploitation of QS trees in 1944 (Martín & Briones 1999a).

Because to the scarcity of *Quillaja* bark saponins, various QS tree sections have been researched as potential saponin sources, and extraction methods have been improved (Gaete-Garretón et al. 2011). It was discovered that the extracts from QS trees' wood and leaves also possessed high levels of saponins (on average 2.58% saponins), and that these saponins had the same compositions and patterns as extracts made just from the bark. In order to stop the deforestation of QS trees in Chile, wood and leaves are now also employed as sources of QS saponin in addition to the bark (Martín & Briones 1999a; Schlotterbeck et al. 2015).

2.3.2. Chemical structure of *Quillaja* saponins

Approximately 100 distinct *Quillaja* saponin structures have been found (Nord et al. 2001), and several naturally occurring saponin structures (Guo & Kenne 2000), as well as novel synthetically generated variants (Fernández-Tejada et al. 2015), have been described in recent years. QS saponins are composed of several triterpene structures (Guo & Kenne 2000), with varied molecular weights (1,500-2,400 g/mol) and hydrophobicity degrees (Tippel et al. 2016). Reversed-phase high-performance liquid chromatography (RP-HPLC) was used to examine the saponin structures in QS bark extract. According to the results, the QS extract contains a variety of important saponin fractions, such as QS-7, QS-17, QS-18, and QS-21, which differ primarily in their monosaccharide composition (Kensil et al. 1991). QS saponins have a basic chemical structure that consists of a hydrophobic triterpene quillaic acid and generally two linked sugar moieties (Higuchi et al. 1986). In general, the basic QS saponin molecules have only one carboxylic acid group (β-d-glucuronic acid) attached at position R1, giving a single chargeable group. A saponin pattern analysis of a QS bark extract exhibited a comparable peak pattern to the commercially available Quil A product, showing that

their extraction source was *Quillaja saponaria* Molina bark (Kensil et al. 1991). The saponin structure QS-18 predominates among the several saponin fractions in QS Molina extracts and may thus be utilized for verification of authenticity (Kensil et al. 1991; Martín & Briones 1999b).

In aqueous or alcoholic extracts of "quillay" bark and wood, at least 100 distinct saponins have been tentatively discovered, with quillaic acid (3,16-dihydroxy-23-oxoolean-12- en-28-oic acid) being the predominant sapogenin. These saponins are bidesmosides, with distinct oligosaccharide and aliphatic acid substituents, glycosylated at the aglycone's C -3 and C-28 locations (Higuchi et al. 1986; Kensil et al. 1991; van Setten et al. 1995, 2000; Guo et al. 1998; Guo & Kenne 2000; Nord & Kenne 2000). Figure 5 shows a generic quillaja-saponin with the aglycone and the aliphatic acid dimer in red.

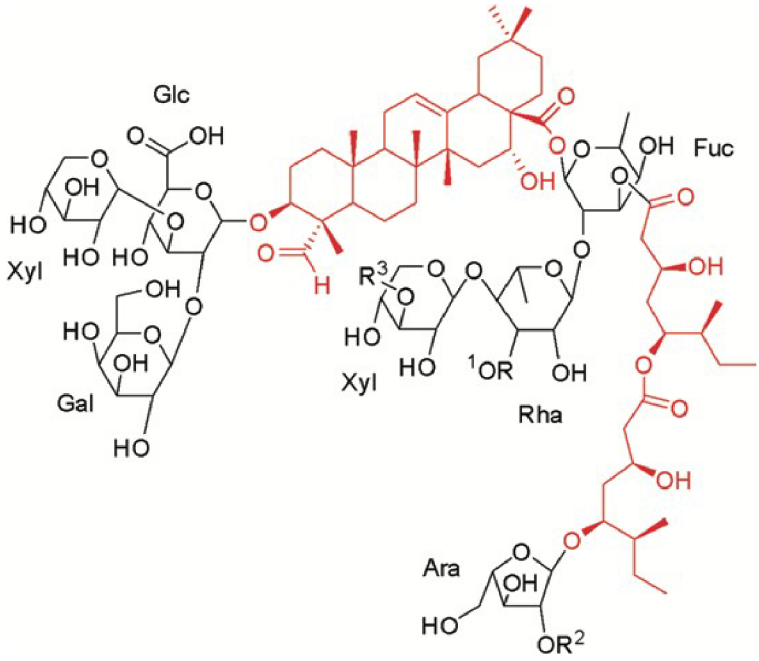

Figure 5. *Quillaja*-saponin with the aglycone and the aliphatic acid dimer in red. Source: Delporte et al. (2021)

Certain biological functions appear to be significantly influenced by the acyl side chain attached at O-3 or O-4 of the fucose residue connected to the C-28 carboxyl group (Fleck et al. 2019). Furthermore, Nord and Kenne (2000) reported saponins with a new fatty acyl group [(S)-2-methylbutanoyl] and an alternative aglycone

(phytolaccagenic acid). Because of their hydrophilic saccharide side chains and lipophilic aglycones, saponins have an amphiphilic structure that accounts for their detergent and foaming qualities (Delporte et al. 2021). Quil-A is a saponin mixture fraction that has been shown in several preclinical investigations (Pham et al. 2006) due to its great potential to promote humoral and cellular immunity. The four saponins that makeup Quil-A are QS-7, QS-17, QS-18, and QS-21, and their structures have been clarified and described (van Setten et al. 1995). Research on QS-21 has been utilized to generate vaccines against herpes, zoster, and malaria, and it is now being employed in these vaccines. This saponin is perfect for use in vaccines against intracellular infections since it generates a robust Th1 response by inducing the generation of cytokines, including IL-2 and INF-γ, as well as antigen-specific antibody responses of both IgG1 and IgG2a (Garçon & Van Mechelen 2011). These saponins' adjuvant properties stem from their capacity to bind to phospholipids and cholesterol, creating spherical colloidal aggregates known as ISCOMs. An adjuvant or immunostimulatory complex (ISCOM), which functions in concert with the antigen to enhance and/or extend the immune response, is frequently included in antigen formulations. ISCOMs are not always immunogenic on their own (Kensil et al. 1991; Kim et al. 2006; Pham et al. 2006).

2.3.3. Biological activity of *Quillaja* saponins

Ebbesen et al. (1976) investigated the biological activity of QS saponins in 1976. The researchers discovered anticancer properties in mice. Several other biological activities of QS saponins have been discovered in recent decades. QS saponins, for example, demonstrated high haemolytic activity against *Staphylococcus aureus*, *Salmonella typhimurium*, and *Escherichia coli* (Hassan et al. 2010) and required low minimum inhibitory concentrations (0.1 mg/ml). There were significant differences in antibacterial activity between saponins derived from guar, QS, *Yucca schidigera*, and soybean (Hassan et al. 2010). QS saponins, in addition to their antimicrobial activity, can modify the surface of bacteria such as Pseudomonas sp. (Kaczorek et al. 2016). Yeast cells were used to investigate additional cell surface modifications. The authors reported that QS saponins increased cell membrane permeability, which can be used to enhance amino acid release (Berlowska et al. 2015). *In vivo* studies in mice previously inflamed with arachidonic acid or 12-O-tetradecanoylphorbol-13 acetate have been used to investigate the anti-inflammatory properties of QS extracts or their specific

constituents such as quillaic acid (Sarkhel 2016). However, when QS saponin extract was administered to mice with carrageenan-induced paw edema, it was slightly less effective in terms of its ability to reduce inflammation than the commercially used drug indomethacin (Sarkhel 2016). QS saponins' most well-known biological activities are adjuvant and immunostimulant properties (Güçlü-Üstündağ & Mazza 2007). Adjuvants are substances added to vaccines to "improve the immune response to an antigen" (Tharabenahalli-Nagaraju et al. 2014). A large number of research studies have focused on the apparent immunomodulatory activity of QS saponins due to their adjuvant activity (Dalsgaard 1978; Kersten & Crommelin 2003; Marciani 2015; E. Walkowicz et al. 2016). The functional groups of QS saponins have been attributed to the adjuvant activity. The central glycosidic linkage is critical, and any changes to this group resulted in a decrease or complete elimination of all adjuvant activity. Changes in saponin molecule stereochemistry resulted in effective adjuvant activity but dose-limited toxicity (E. Walkowicz et al. 2016). Damage to the cell membrane of the midgut epithelium of aphids has been attributed to the insecticidal activity of QS extract (De Geyter et al. 2011). In the case of fish, QS saponins demonstrated dose-dependent insecticidal activity, with 46% and 100% mortality rates of aphids when 1 and 3 mg/ml of QS saponins were added to their diet (De Geyter et al. 2011). According to a report from a World Health Organization (WHO) meeting, QS saponins are promising agents for immunostimulant and adjuvant applications. According to Kenney (et al. 2002), clinical trials in humans will be conducted in the future (Kenney et al. 2002). To date, few studies have been conducted to investigate the biological activity of QS saponins on human cells using an *in vitro* model (Roner et al. 2007) and *in vivo* studies using animals (Tam & Roner 2011). At a concentration of 0.01 mg/ml of QS saponin extract, antiviral activity, such as inactivation of 50% of vaccinia virus in human cells, has been reported. When 0.9 mg/ml of QS saponin extract was added to human cells, the cell death rate was 50%. According to the authors, QS saponin extract disrupted the viral envelope and capsid proteins, providing antiviral activity at low concentrations below human cell toxicity levels (Roner et al. 2007). QS saponin administration inhibited virus-host attachment by disrupting the cellular virus membrane, effectively reducing a rhesus rotavirus infection that would have caused diarrhoea in mice and thus decreasing the mortality of mice infected with the rhesus rotavirus (Tam & Roner 2011).

2.3.4. Saponins of *Yucca* genus

Yucca species have long been utilized in traditional medicine, particularly by Native Americans, but also by people all around the world (Patel 2012). As a result, studies conducted on this genus have demonstrated that the plants have a high concentration of bioactive steroidal saponins (Joanne et al. 2007). Plant-derived secondary metabolites known as steroidal saponins have a complicated two-part structure and a high molecular weight. One of these components, referred to as the aglycone or sapogenin, is hydrophobic and is made up of 27 carbon atoms grouped into five or six rings, denoted A–F. They fall into one of two categories: spirostane or furostane (Figure 6), depending on the type of aglycone attachment at C-22. The hydrophilic moiety, which is the other one, is often composed of sugar residues that are connected by O-glycosidic linkages between distinct monosaccharides (Sparg et al. 2004; Vincken et al. 2007). Furthermore, two saponins of the cholestane class have been identified specifically in the Yucca genus (Skhirtladze et al. 2011).

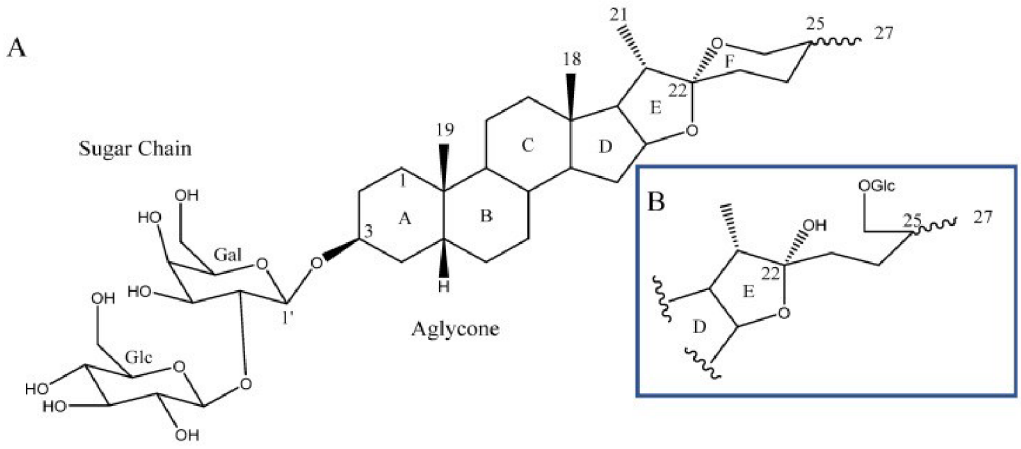

Figure 6. Structure of a spirostanic saponin (A) C-22 to C-27 carbons of a furostanic saponin (B). Source: Jiménez et al. (2021)

Currently, some *Yucca* species and their saponins have been classified as generally recognized as safe (GRAS) by FDA - the US Food and Drug Administration (Piacente et al. 2005; Montoro et al. 2010; Patel 2012). As a result, several extracts and products made from these plants have been sold as moisturizing agents, food supplements, and parapharmaceutical complements. Worldwide, YS products are

produced by a number of companies, including American Extracts, Naturex, and BAJA Yucca. Several investigations have also examined the saponins found in these plants, revealing characteristics including cytotoxicity, phytotoxicity, antifungal, molluscicidal, and anti-inflammatory effects (Diab et al. 2012; Pérez et al. 2013; Yokosuka et al. 2014).

Numerous reviews have been published on steroidal saponins from the Agavaceae family, which includes the *Yucca* genus. However, no work has been published that focuses solely on this genus.

2.3.5. Biological activity of *Yucca* saponins

Certain extracts from *Yucca* species have demonstrated antifungal activity against a wide range of yeasts and fungi, including those from *Y. gloriosa* flowers or dried leaves (Favel et al. 2005; Kemertelidze et al. 2009), as well as YS stems (Miyakoshi et al. 2000). As a result, these extracts are a good fit to be employed as food additives or preservatives (Uematsu et al. 2000). YS extract is commercially accessible and has been classified as GRAS by the FDA (USA) (Piacente et al. 2005). It also appears on the "List of Existing Food Additives" in Japan, allowing it to be used as a human dietary additive (Uematsu et al. 2000). Furthermore, it has been suggested that a few YS saponins and extracts can enhance the immunological response of broilers (Sun et al. 2009) and the health of aquatic creatures [(Fayed et al. 2019; Paray et al. 2021)]. Additionally, giving YS extract to horses or ruminants reduces the growth of protozoa and Gram-positive bacteria, which are sometimes linked to colitis and other processes that result in inflammatory illnesses (Cheeke 2000). Investigations have also been conducted on the saponins that were isolated from various *Yucca* extracts. Tannins from *Y. glauca* (Yokosuka et al. 2014), *Y. desmetiana* (Eskander et al. 2013), and YS (Qu et al. 2018) have been reported to exhibit intriguing cytotoxic properties against a range of cancer cell lines. Additionally, studies on pure saponins and epimer combinations on the C-25 position in *Y. gloriosa* (Favel et al. 2005), *Y. elephantipes* (Zhang & Popovich 2009), and YS (Miyakoshi et al. 2000) have demonstrated antifungal action, with highly encouraging activity levels.

Some of the saponins described in the genus Yucca have been evaluated for bioactivities such as anti-inflammatory properties after being isolated from other

species. Inflammation (Franco Ospina et al. 2013), a protective physiological reaction to unpleasant stimuli, is a very complicated process that involves numerous cells, communication channels, and chemicals. Although inflammation is a normal and useful process, an excessive inflammatory response can cause long-term harm and undesirable effects. Some saponins have been tested against processes involved in the inflammatory response, such as nitric oxide (NO) release, inflammatory interleukin production (Wang et al. 2017c), immune system cell recruitment (Park et al. 2015), and inhibition of specific metabolic pathways (Yuan et al. 2016), and in some cases, they showed promising results.

2.4. Cytotoxicity of *Yucca* and *Quillaja* saponins

One of the most frequently investigated activities of natural substances is cytotoxicity, which is commonly reported for *Yucca* and *Quillaja* saponins. In most cases, these compounds are tested for their potential to cause damage to cancer cell lines. Such damage can occur through various pathways or modes of action, the most common being the regulation of cellular apoptotic pathways (Yokosuka et al. 2014). In terms of QS toxicity, there have been few research published. The research that is available focuses on preclinical toxicity evaluation. Rönnberg et al. published one of the first studies investigating the toxicity of QS and its components in 1995 (Rönnberg et al. 1995). This study evaluated the toxicity of crude QS extract and QH-A (an iscom-forming saponin with low adjuvant activity and low toxicity), QH-B (strongly adjuvant active but highly toxic), and QH-C (adjuvant active but considerably less toxic than QH-B) purified triterpenoid components in WEHI cells (immature B-lymphocytes of Mus musculus). The study of the lytic activities of QS which is responsible for the local reactions after injection, revealed that QH-A was safer than QH-B and QH-C, inducing hemolysis only at ten times the concentration. Furthermore, QH-B and QH-C did not inhibit RNA or protein production, indicating that these are not the primary toxicity targets (Marciani 2015). Despite its significant adjuvant action, QH-B is the most toxic triterpenoid saponin, which limits its use *in vitro* and *in vivo* (Rönnberg et al. 1997).

The concentration of QS saponins administered is important for most applications because QS saponins can be toxic at higher concentrations (Marciani 2015; E. Walkowicz et al. 2016). For example, adding 450 mg/kg QS saponins to a carp dry

diet (26 μg QS saponin per carp) for four weeks had no toxic effect on the tested fish (Serrano 2013). Acute toxicity against olive flounder was determined in a dose-dependent manner, with a mortality rate of 5% at low concentrations (16 μg QS product/fish). When the fish were given injections of 500 μg QS saponin extract, the mortality rate increased to 95% (Tharabenahalli-Nagaraju et al. 2014). The dead fish were examined and found to have damaged kidney tubule epithelium cells and red blood cells (Tharabenahalli-Nagaraju et al. 2014).

Arabski et al.(2012) investigated the *in vitro* toxicity of QS using the CHO-K1 cell line. Saponins were found to dramatically promote early apoptosis in this cell line at dosages ranging from 12 to 50 μg/ml. Minor doses were ineffective in lysing exposed erythrocytes, revealing the minimum concentration required to intersperse with cell membranes in order to lyse them. Tam & Roner (2011) conducted an *in vivo* study on the acute toxicity of QS extract. The researchers administered 50 μL of saponin extract to newborn mice. The concentrations varied from 0 to 0.5 mg for each mouse. The animals were treated for seven days and monitored for two months. The 0.0375 mg/mouse dose resulted in more than 50% mortality, hence the extract's LD_{50} was determined to be 0.0325 mg/mouse. The weight examination revealed that there was no short-term health impact in mice that survived saponin treatment. On the other hand, excessive amounts of the extract killed the mice within 24-36 hours, indicating a substantial shift in the gastrointestinal tract's membrane environment that caused cells to rupture.

In the case of YS cytotoxicity test was performed *in vivo* by Wisløff et al. (2008) on lamb. YS juice in doses of 1.5 g (63 mg sapogenin) and 3.0 g (126 mg sapogenin) per kg live weight was administered intraruminally to 30 lambs for 21 days to investigate whether the saponins in YS were toxic to lambs. Twelve lambs died or had to be euthanised. The main pathological findings in the diseased lambs were acute tubular necrosis in the kidneys, dehydration, and watery content in the gastrointestinal tract.

2.5. Saponins in feed additives

Plant-based extracts of animal health interest can be derived from a variety of trees, shrubs, or fruits. QS, called the Chilean soapbark tree, is an example of a plant high in polyphenols and triterpenoid saponins. This plant is frequently applied in conjunction with YS, a plant high in steroidal saponins with similar properties and chemical composition (Sparg et al. 2004). Because of the chemical characteristics of saponins, feed additives, including a mixture of QS and YS, have been commercially marketed for use with companion and production animals. For more than 20 years, the additive has been sold for use in farm animals in the US, South America, Asia Pacific, and some other regions. It has been demonstrated to be beneficial in lowering environmental ammonia, litter moisture, and footpad dermatitis, and it also helps in minimizing the adverse effects of intestinal challenges in chicken production (Augustin et al. 2011; Onbaşilar et al. 2014). YS and QS saponin-containing extracts are currently used as dietary additives for livestock and companion animals, primarily for ammonia and odor control (Killeen et al. 1998). YS has ammonia binding activity. When added to the diet, YS saponins pass through the digestive tract and are excreted in the faeces. In faeces, YS components bind to ammonia and prevent its release into the air. Some companies, such as the Czech company Addicoo group s.r.o., use saponin substances in an attempt to reduce the intensity of nitrogen waste decomposition. QS and YS biomass are known for reducing cell inflammation in broiler feed, leading in increased feed efficiency and growth performance (Bafundo et al. 2021). Bioactive substances, saponins, and polyphenols derived from a blend of QS and YS have been shown to improve the integrity of biological membranes, provide antioxidant activities (Sparg et al. 2004), and reduce coccidiosis-related damages (Bafundo et al. 2020). Furthermore, it has been found that the additive improves food digestibility, increases intestinal villus height, and reduces oocyst cycling and necrotic enteritis (Bafundo et al. 2021). The need for plant-based feed additives has increased in chicken production as the removal of antibiotic growth promoters (AGP) from diets has become an important goal for broiler breeders. In AGP-free chicken production, additives, including saponins and polyphenols, have been demonstrated to increase broiler intestinal health and BW gain or feed efficiency, especially if combined with complementing management and biosecurity approaches. As a result, QS and YS products have been advised to

supplement anticoccidial therapies for broilers exposed to intestinal challenge (Bafundo et al. 2020).

2.6. Gastrointestinal tract

The gastrointestinal tract (GIT) is the organ system that enables the intake and processing of food, absorption of nutrients and excretion of indigestible residues. The digestive system is divided into the alimentary canal and adjacent organs that secrete digestive enzymes and fluids that aid the digestive process. Each organ serves a distinct and irreplaceable purpose. The oral cavity is the first part of the digestive system, followed by the pharynx and esophagus. These organs are responsible for the intake and grinding of food, as well as its moistening and softening via saliva. The meal enters the stomach after passing via the throat and oesophagus, where enzymes from gastric juice and an acidic pH allow mechanical and chemical digestion. Thus, the higher sections of the GIT do not play a direct nutrient absorption function, but rather help to commence food digestion. The alimentary canal passes through the duodenum, jejunum, and ileum that make up the small intestine (*intestinum tenue*), and the large intestine (*intestinum crassum*) that splits into the appendix (*intestinum caecum*), which is a worm-like appendix, the ascending, transverse, descending esophagus *(colon ascendens, transversum, descendens, sigmoideum*), and culminates in the anus, the rectum's outlet. The liver (*iecur),* pancreas, and gallbladder (*vesica fellea*) are the auxiliary organs (Vertzoni et al. 2019).

The main part of the digestive process begins in the stomach, which can be divided into four parts: the fundus, the body, the antrum and the pylorus. The total resting volume of the stomach is about 1 litre, but it can increase up to 1.5-4 litres (Norton et al. 2014). The proximal stomach (fondus and body) serves as a storage for undigested material, whereas the distal stomach (antrum) crashes and blends solid meals. The stomach has three purposes: mixing, storage, and emptying (Kong & Singh 2008). Gastric motility, variations in force, pressure, and changes in gastric flow all impact the pace of food digestion in the stomach (Norton et al. 2014). On average, the stomach secretes 2-3 litres of gastric fluid (pH 2), which contains salt, stomach acid (hydrochloric acid), and digesting enzymes (pepsin, lipase). Because these gastric secretions are aggressive, the stomach wall is protected by a mucous layer that shields it

from self-damage caused by the acids in the digestive juices. This layer is created by foveolar cells, which are found in the pits of the stomach and produce mucus and bicarbonate ions. Parietal cells are in charge of secreting hydrochloric acid. Primary gastric cells generate the inactive precursor pepsinogen, which is activated by hydrochloric acid to become pepsin, as well as gastric lipase, which is responsible for 10-30% of dietary triglyceride breakdown (Bornhorst & Paul Singh 2014). Gastric juices enter the gastric bolus, dilute it, and guarantee homogeneity and mixing with stomach contractions (Kong & Singh 2008). The presence of food in the stomach causes cells to secrete gastric juice (Boland 2016). The contents of the stomach are changed into a multiphase mixture known as chyme by the stomach's contractions and gastric juice. Separate stages of aqueous solution, solids, and lipids are present in chyme (Kong & Singh 2008). The chyme is return back to the stomach's body by the peristaltic waves, where the produced gastric fluids fully mix and emulsify it. Between 0.2 and 1.89 newtons of force can be applied by the contraction forces (Norton et al. 2014). The duodenum, jejunum, and ileum make up the small intestine, which is where the majority of digestion occurs. The small intestine performs absorptive, excretory, and digesting tasks. The vast number of mucosal folds with villi enlarges the surface of the small intestine considerably (Figure 7). These villi contain absorptive cells that can absorb substances. These cells are characterized by having a rim of microvilli which increases their surface area. The rim of microvilli also contains glycocalyx which contains digestive enzymes. The epithelial cell line is on the subjacent cell membrane. This subjacent layer includes the mesenchymal lamina propria, which contains blood vessels (Gelberg 2014).

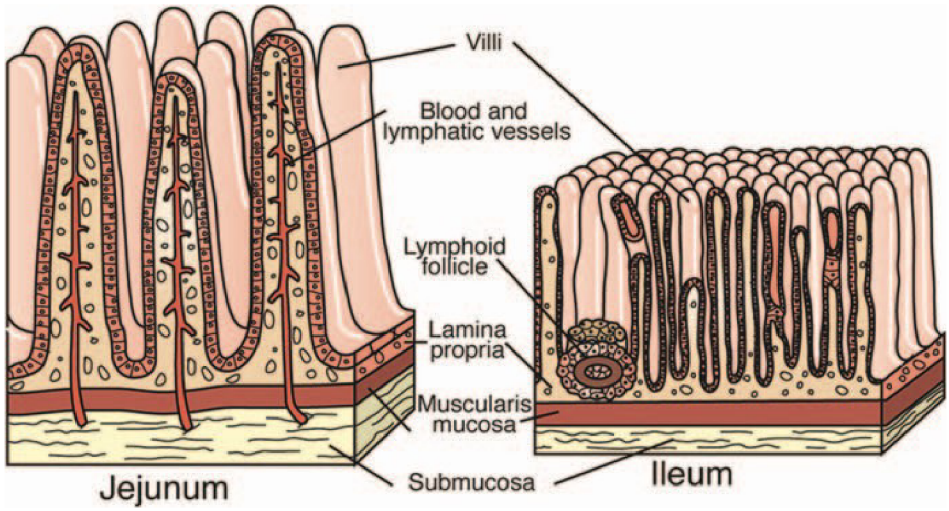

Figure 7. Schematic diagram of the anatomic and histologic organization of the digestive tube. Source: (Kierszenbaum 2002).

The ileum and jejunum absorb digested nutrients, whereas the duodenum handles the majority of digestion. Digestive enzymes and pancreatic enzymes (lipases, amylases, and a complex combination of proteases) generated by the small intestine's inner wall collaborate to break down meal components. The pancreas secretes bicarbonate into the duodenum to maintain an appropriate pH for these enzymes of around 6-7. These enzymes facilitate the digestion of lipids into free fatty acids and 2 - monoglycerides, proteins into dipeptides, tripeptides, and amino acids, and carbohydrates into monosaccharides (galactose, fructose, and glucose). Additionally arriving at the duodenum, bile from the gallbladder facilitates the emulsification of fats into tiny droplets, which in turn stimulates the activity of pancreatic lipase and plays a significant role in lipid digestion. Undigested material is transported by motor activity from the small intestine to the large intestine, where it undergoes fermentation (Li et al. 2020). Adjacent to the ileum, the big intestine is comparatively short and devoid of villi. The reabsorption of water, minerals, and vitamins is significantly aided by the large intestine (Denbow 2015).

Apart from its involvement in food intake and digestion, the GIT also plays a vital role in the immune system and in defending the body against infections. The gut wall creates a strong physical barrier that keeps germs from entering the circulation and subsequently reaching other organs. Additionally, the GIT's resident microorganisms

also act as a barrier to protection (Cordonnier et al. 2015). The human body and microbes are always in symbiosis. The term "microbiota" refers to the group of microbes that inhabit the human body. Since the bacteria in the human body are dispersed unevenly, the skin, oral cavity, respiratory tract, urogenital tract, and gastrointestinal tract microbiota are often distinguished from one another (Xu & Knight 2015).

2.6.1. Microbiota of the gastrointestinal tract

In the human digestive system, there are over a thousand different kinds of microorganisms and 10^{14} different types of bacteria. Certain sections of the GIT have a very diverse mix and quantity of bacteria. The stomach has the fewest bacteria because of the low pH of gastric liquid and proteolytic enzymes, which make it difficult for microbiota to grow there. There are only 10^2 colony forming units (CFU) per milliliter, with members of the *Streptococcus*, *Stomatococcus*, and *Sarcina* genera among them. Approximately 10^4 CFU/ml live in the small intestine. Gram-positive aerobic bacteria, such as *lactobacilli, streptococci*, and *staphylococci*, predominate in the proximal section of the small intestine, whereas gram-negative enterobacteria and other anaerobic bacteria predominate in the distal region (Sender et al. 2016). This is the human body's most abundant and diversified microbial ecosystem. *Bacteroides*, *Clostridium*, and *Bifidobacterium* are the most common bacteria in the large intestine, with *Escherichia* and *Lactobacillus* being less common. The large intestine also contains *Candida* and *Saccharomyces* yeasts, tiny fungus, and *Archea* (Newton et al. 2013). The GIT microbiota's species makeup varies depending on a number of variables. Genetic predisposition, age, surroundings, way of life, and medications used all have an impact (Ashaolu et al. 2021). For instance, taking broad-spectrum antibiotics causes the populations of bacteria that promote health (such as *Lactobacillus* and *Bacteroides*) to decline, as does their metabolic activity (Newton et al. 2013). A healthy, high-fiber diet, in turn, supports the maintenance of normal gut flora (Rajoka et al. 2017).

It has long been believed that microbes invade the human body during birth, meaning the fetus is completely sterile before to birth (Xu & Knight 2015). Until recently, infections of the placenta, amniotic fluid, and/or premature delivery were the most common causes of bacterial colonization of the fetus (Walker et al. 2017).

However, it has been found that bacteria from the genera *Enterococcus, Lactococcus,* and *Leuconostoc* are present in the placenta, intrauterine fluids, and umbilical cord blood (Aagaard et al. 2014). The fetus is continuously surrounded by amniotic fluid, which mediates bacterial colonization before birth *(in utero)*. The genera *Streptococcus* and *Enterococcus* are among the bacteria that are colonizing the fetus in pregnancy (Nagpal et al. 2016). The manner of birth is an essential stage in the formation of the baby's microbiota (Walker et al. 2017). The microbiota of babies delivered by caesarean section is similar to the skin, much smaller, and contains bacteria from the orders *Bacillales* and *Lactobacillales*. In contrast, the microbiota of babies delivered vaginally resembles that of the mother and is represented by bacteria of the orders *Bacteroidales* and *Enterobacteriales* (Akagawa et al. 2019). The sort of food a child receives affects how their gut microbiome develops (Yasmin et al. 2017; Akagawa et al. 2019) as well as regional differences and hygiene norms (Selma-Royo et al. 2021). According to Akagawa et al. (2019), there is a significant correlation between the host's health and the makeup of the microbiota. The gut microbiota assists the host's immune system, ferments indigestible carbohydrates, provides energy in the form of short-chain fatty acids, and protects the organism from infections. Dysbiosis, or a qualitative shift in the microbiota's typical species composition, has been connected to the onset of a number of illnesses.

Taking probiotics is one method of preserving the GIT microbiota's typical qualitative and quantitative composition (da Silva et al. 2021).

2.6.2. Changes in the gut microbiota composition

The composition of our gut microbiota is greatly influenced by the type of diet we consume. Our microbiome changes completely within 24 hours if we consume a fully plant-based or exclusively meat-based diet. The recovery period might last up to 48 hours (David et al. 2014). Humans, like animals, have proven that when there is substantial stress in the body, such as inflammation, abrupt alterations in the gut microbiota can occur within a single day (Singh et al. 2017). Other variables, such as antibiotic usage, dietary composition, and physical stress, may contribute to gut dysbiosis in the microbiome. Dysbiosis is likely to disrupt the normal operation of the gut microbiota and digestion. This situation promotes the growth of specific bacteria,

particularly pathobionts, and the uncontrolled creation of metabolites that can be detrimental to the host, resulting in a variety of disorders (Kho & Lal 2018).

2.6.3. The role of gut microbiota

The human microbiota, particularly the gut microbiota, has been dubbed an "essential organ" (O'Hara & Shanahan 2006) since it contains nearly 150 times more genes than the total human genome (Ursell et al. 2014). Significant advances have proven that the gut microbiota is engaged in basic human biological processes, such as altering the metabolic phenotype, controlling epithelial development, and impacting innate immunity (Savage 1977; Whitman et al. 1998; Ley et al. 2006a). The human microbiome has been linked to a number of chronic illnesses (Figure 8), including cirrhosis, hepatocellular carcinoma, diabetes mellitus, metabolic syndrome, obesity, alcoholic liver disease (ALD), nonalcoholic fatty liver disease (NAFLD), inflammatory bowel disease (IBD) and metabolic syndrome (Ley et al. 2006b; Wang et al. 2016).

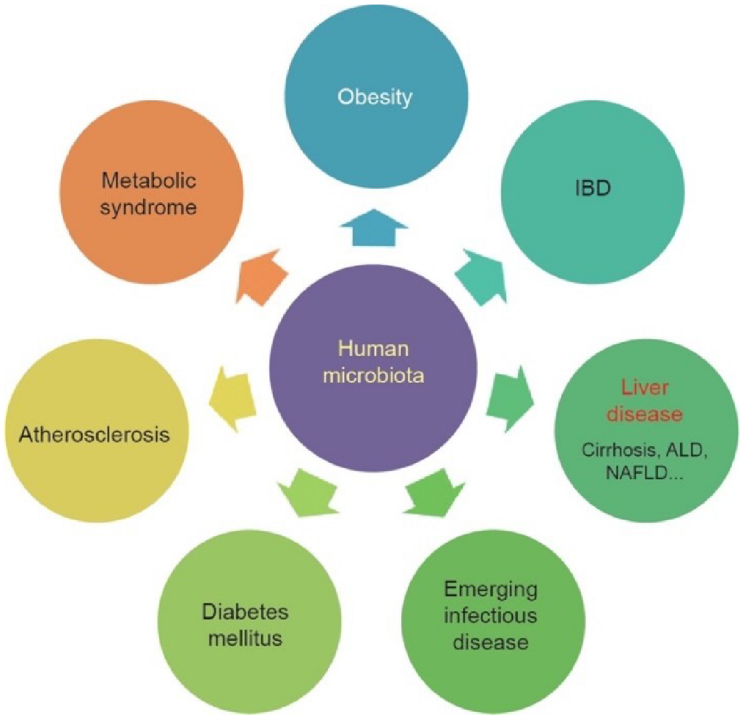

Figure 8. Human microbial symbiosis has a close relationship with diseases of different systems. Source: Wang et al. (2017)

2.7. Probiotics

The term "probiotics" has many definitions. Nonetheless, they are primarily defined as naturally occurring live bacteria found in the human gastrointestinal system that, when present in appropriate amounts, benefit human health. Probiotics are defined by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) as live microorganisms that, in specific amounts, may benefit human health. They have the power to control the environment in the intestines and stop several illnesses (McFarland 2015). Foods supplemented with modulating microbes are referred to as probiotics (Matsumoto et al. 2010). The most significant species utilized as probiotics include specific species of yeast (*Saccharomyces* spp.), a subspecies of *E. coli*, *Bifidobacterium* spp., and *Lactobacillus* spp. (Hudson et al. 2016). Probiotics have the ability to positively control gut health and may potentially be used to cure or prevent inflammatory bowel disease (Shen et al. 2014). Probiotics also lessen symptoms of gastrointestinal intolerance (del Campo et al. 2014), limit pathogen adhesion in the intestinal mucosa (Collado et al. 2007), and reduce ascites and abdominal distension in individuals with chronic liver disease (Liu et al. 2010). Many mechanisms (Figure 9) allow probiotics to prevent pathogenic bacteria from adhering to the intestinal mucosa, including higher adhesion than pathogens, enhanced integrity of the epithelial barrier, and generation of antimicrobials (Hidalgo-Cantabrana et al. 2014; Yousefi et al. 2019)

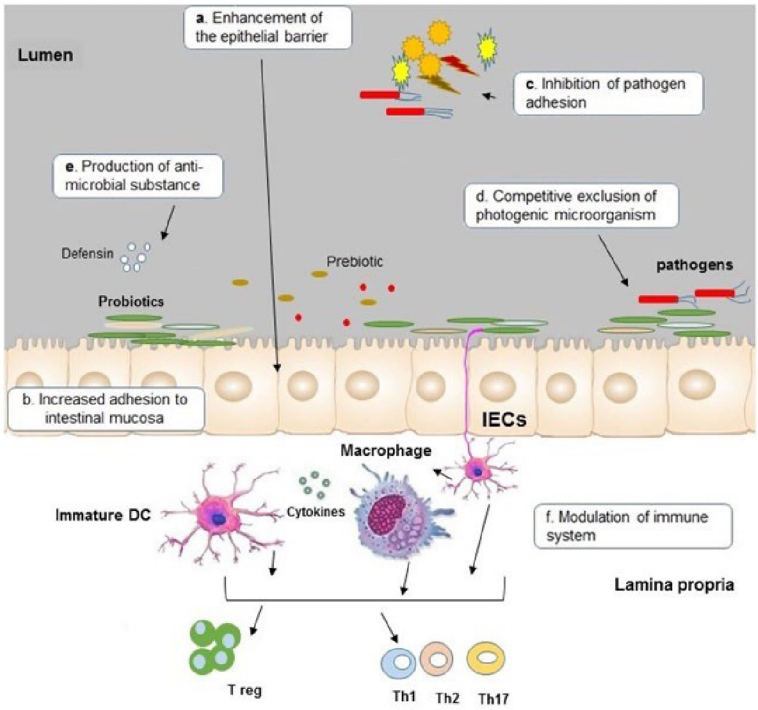

Figure 9. Major mechanisms of action of probiotics. DC: denditic cells, IEC: intestinal epithelial cells. Source: Yousefi et al. (2019)

2.7.1. *Lactobacillus* spp.

The genus Lactobacillus comprises around 170 species of facultative, anaerobic, catalase-negative, Gram-positive, non-spore-forming rods that are taxonomically placed in the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, and family *Lactobacillaceae* (Kleerebezem & Vaughan 2009). For ages, cultures have employed lactobacilli (LAC) to produce fermented meals derived from both plants and animals. They are currently among the most often utilized probiotically active microorganisms. The age of the host, the health of the gut, and other factors all affect how many LAC are present in the gastrointestinal system. Adults' feces include the majority of native *Lactobacillus* species, including *L. gasseri, L. reuteri, L. crispatus, L. salivarius,* and *L. ruminis*. When it comes to babies, *L. plantarum, L. salivarius, L. rhamnosus, L. paracasei, L. fermentum, L. gasseri, L. delbrueckii*, and *L. reuterii* are the most common representatives (Lebeer et al. 2008). The gastrointestinal tracts of humans are most frequently inhabited by *L. casei*, *L. plantarum, L. fermentum*, and *L. rhamnosus*. Most common in the stomach are *Lactobacillus antri, Lactobacillus gastricus, Lactobacillus calixensis, Lactobacillus reuteria, and Lactobacillus ultunensis* (Turroni

et al. 2014). A sizable and consistent amount of LAC species, which provide protection against pathogenic infections, are present in the female urogenital tract in health. The species that are most often represented include *L. crispatus, L. gasseri, L. jensenii, L. vaginalis*, and *L. iners* (De Gregorio et al. 2014). LAC have a major positive impact on the growth of their hosts, affecting their size and weight (Schwarzer et al. 2016; Hamdan et al. 2016). Certain members of this genus have the ability to increase the bioavailability of macro- and micronutrients that are not metabolized by the host. This is accomplished by altering the physiology of the gut by controlling the production of growth factors such calcium, B vitamins, and spermidine (Turpin et al. 2010). LAC have the ability to produce B vitamins that are necessary for the host, including B12, B9 (folate), and B2 (Zhang et al. 2018). Probiotic LAC can be utilized to treat infectious disorders brought on by rotaviruses, herpes simplex virus type 1, and adenoviruses (Todorov et al. 2008; Yang et al. 2015). It has been shown that LAC has fungistatic attributes, meaning they may successfully fight serious fungal illnesses, especially those affecting the digestive system (Black et al. 2013; Cortés-Zavaleta et al. 2014).

2.7.2. Adhesion of probiotics on intestinal cells

Mammals' gastrointestinal tracts are coated with mucus, a viscoelastic gel that coats, and divides the intestinal epithelium from the lumen contents. The mucus covers the large intestine in two layers; the outer layer acts as a colonization environment for bacteria, while the inner layer keeps them away from the epithelial surface. Thus, the inner layer in its normal state does not contain any microorganisms (Johansson et al. 2011). Mucus thickness varies throughout the system, with the colon having the thickest mucus. This mucus provides a rich supply of nutrients utilized in the metabolism and proliferation of bacteria, and it also traps and transports germs (Kim & Ho 2010). Mucins (MUC) are the primary constituent of mucus, which are generated, stored, and released by goblet cells. Glycans make up 80% of the molecular weight of glycoproteins called mucins. There are now 20 human MUC genes in the MUC gene family; some of them are categorized as mucins that create mucus on their own, while others are categorized as mucins that are sessile to the membrane (Desseyn et al. 2000; Ringot-Destrez et al. 2018). This mucus layer also includes glycolipids, electrolytes, and immunoglobulins in addition to mucin glycoproteins; together, these components are called the glycocalyx (Bron et al. 2011). In the first phase, non-specific hydrophobic

physical linkages like hydrophobic interactions regulate bacterial adhesion to the intestinal surface. In the second phase, particular cell wall components exert adhesion (Haddaji et al. 2015). Some lactic acid bacteria are more hydrophobic and adhere better when certain surface proteins, such as cell wall-anchored proteinases, are present (Muñoz-Provencio et al. 2012; Zhang et al. 2015; Radziwill-Bienkowska et al. 2017). Bacterial adherence in the gut is significantly influenced by the presence of adhesins in the bacterial cell wall (Popowska et al. 2017). Fimbriae and pili (thin protein extensions seen in bacterial cells) also increase adherence. Type IV pili have been extensively studied in Gram-negative bacteria. These features provide bacteria with an edge in the colonization of mucosal surfaces (Hospenthal et al. 2017). According to Piepenbrink & Sundberg (2016), this form of pili is also seen in Gram-positive bacteria like *Bifidobacterium*.

In vitro experiments with mucin adsorbed on abiotic surfaces and human tumorigenic cell lines such as Caco-2 and HT29 to imitate intestinal epithelial cell adhesion are routinely used to assess probiotic bacteria adherence (Lebeer et al. 2012; Monteagudo-Mera et al. 2012; Tuo et al. 2013; Garriga et al. 2015). The usage of epithelial cell lines is particularly beneficial for identifying adhesion processes and molecules. Wang et al. (2017b) employed HT29 cell lines to find a novel surface layer protein, choline A-binding protein, which is required for adhesion of a novel probiotic strain, *Lactobacillus salivarius* REN. Characterization of adhesive molecules and their genes may be important for the construction of vectors that enhance the adhesive potential of other probiotic strains (Zhang et al. 2015). Although studies conducted *in vitro* are important for understanding adhesion processes and identifying probiotic candidates that would attach *in vivo*, it is challenging to apply these findings to the human gastrointestinal system. *In vitro* investigations ignore a multitude of aspects such as peristaltic motions, the host immune system, or competition among resident bacteria (Park et al. 2015). Probiotic strains are generally discovered in faeces samples in *in vivo* research since this approach is non-invasive (Mai et al. 2017). Although the yield of probiotic bacteria in faeces indicates resilience to the difficult conditions of the human gastrointestinal system, it does not reveal the total amount of bacteria adhering to the intestinal mucosa. In this sense, investigations using invasive techniques (such as biopsy) may give information on the underlying processes of probiotic adherence in the colon. Zmora et al. (2018) conducted a human investigation on a group of healthy

volunteers who received a probiotic supplement twice daily for four weeks. Finally, stool samples were taken, and participants underwent deep endoscopy and colonoscopy as a result of collecting straight from the upper and lower gastrointestinal tracts. The scientists discovered that the capacity to colonize and attach to intestinal epithelial cells differed across people. This lends credence to the idea that the resident microbiota has a significant influence on probiotic bacterium adherence, and that developing *in vitro* models with normal microbiota might give a more realistic chance to study the adherence characteristics and processes of probiotic strains. Although germ-free animals and genobiotics are valuable tools for researching probiotic effects in the gut, host genetics and translation to humans are still limited. New technology *„gut-on-a-chip“* models may open up new avenues for studying the processes of in vitro bacteria adhesion to diverse human gut cell populations and peristaltic movements (Jalili-Firoozinezhad et al. 2019).

3. Aims of the Thesis

The aim of the theses was to determine the biological properties of saponin-rich extracts from *Quillaja Saponaria* (QS) and the combination of *Quillaja saponaria* and *Yucca schidigera* (QY).

The specific objective of this study was to digest *Quillaja saponaria* extract and the extract of the combination of *Quillaja saponaria* and *Yucca schidigera* (in a 50:50 ratio) using an *in vitro* digestion model and subsequently evaluate:

- the toxicity of the digestates and
- the effect of the digestates (and their fractions) on the adhesion of selected members of the gut microbiome to the epithelial cells.

The main hypothesis of this study was that QS and QY extracts would affect the adhesion of selected lactobacilli species to the epithelial cell line.

4. Methods

At first, QS and QY extracts were digested using *in vitro* static model INFOGEST (Brodkorb et al. 2019) *in vitro* digestion model. To select appropriate concentrations of QS and QY extracts, cytotoxicity was tested. For the cytotoxicity, the cell monolayers (HT29 and Caco-2) were tested separately. Subsequently, an adhesion experiment was performed. LAC (3 species of lactobacilli) were stained with fluorescein isothiocyanate dye. A medium containing stained LAC and QS extract, QY extract was pipetted onto the cell monolayer (co-culture of HT29 and Caco-2 cells). Each LAC species *(Lacticaseibacillus rhamnosus, Lactobacillus gasseri, Lactobacillus brevis*) was tested at three different concentrations (300, 150, 75 μg/ml) of the test samples. Fluorescence was then measured on reader Infinite M200 TECAN (FITC method). The percentage of adhesion was calculated in Excel.

4.1. Plant material

In the case of QS extract the pure liquid extract from the biomass of QS was used. The extract was purchased from a certified producer in Chile. The material comes from the Los Angeles area, where there are extensive QS forests. *Quillaja* forests cover 800 000ha. In this area, only 35% of the biomass can be harvested at a time, and the next harvest can take place in 5 years, so the forest can then quickly regrow. The harvested biomass is then chopped into small pieces and the saponin extract is obtained by subsequent maceration. Unlike QS extract, obtaining QY extract is complicated because the trunks used to obtain the extracts take a very long time to grow. Although the harvesting of the plant material is regulated, illegal destruction of the plantations occurs because the regulation is difficult to enforce in remote desert areas. Attempts to establish plantations have encountered financial difficulties and the long-term nature of these projects. Both QS and QY extracts were obtained from a certified producer.

4.2. Digestion of the QS and YS sample

Using the static *in vitro* static digestion model INFOGEST (Brodkorb et al. 2019), the QS and QY (mixture of *Quillaja saponaria* and *Yucca schidigera* 50:50) samples were digested in oral, gastric, and intestinal phases. The result was a digestate of the QS and QY samples, which were frozen and subsequently subjected to further analyses.

4.2.1. Preparation of solutions and digestive juices

For each phase of the experiment, electrolyte solutions of digestive juices of appropriate composition were prepared and enzymes with appropriate activity were used (Table 1). Salivary (SSF), gastric (SGF) and intestinal (SIF) simulated fluids were prepared. The compounds listed in table 1 were used to prepare these solutions, which were made up to a final volume of 400 ml using distilled water.

Table 1. Preparation of solutions and digestive juices

Chemicals	Concentration of stock solutions		SSF	Final salt concentration in SSF	SGF	Final salt concentration in SGF	SIF	Final salt concentration in SGF
	g/l	mol/l	ml	mM	ml	mM	ml	mM
KCL	37.3	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH_2PO_4	68	0.5	3.7	3.7	0.9	0.9	0.8	0.8
$NaHCO_3$	84	1	6.8	13.6	12.5	25	42.5	85
NaCl	117	2	–	–	11.8	47.2	9.6	38.4
$MgCl_2*(H2O)_6$	30.5	0.15	0.5	0.15	0.4	0.12	1.1	0.33
$(NH_4)_2CO_3$	48	0.5	0.06	0.06	0.5	0.5	–	–
HCl	–	6	0.09	1.1	1.3	15.6	0.7	8.4
$CaCl_2(H_2O)2$	44.1	0.3	0.025	1.5	0.005	0.15	0.04	0.6

Chemical producers: KCL- Sigma Aldrich, KH_2PO_4- Lachner, $NaHCO_3$ - Lachner, NaCl- Lachner, $MgCl_2*(H2O)_6$ - VWR, $(NH_4)_2CO_3$- Sigma Aldrich

4.2.2. Phases of digestion

Briefly: 5 g of QS and QY samples were weighed into a 50 ml Falcon type tube. The samples were mixed in oral phase with 3.5 ml simulated salivary juice (SSF), amylase (Sigma Aldrich) with 75 U/ml activity, $CaCl_2(H_2O)_2$ solution (VWR) with a molar concentration of 1.5 mM and water to obtain a bolus with a paste-like consistency. The mixture was made up to 1:1 with distilled water. Due to the properties

of the saponins contained in the QS and QY samples, the samples formed a foam in the tube. Subsequently, the samples were incubated for 2 minutes at 37 °C.

Before the gastric phase, SGF was enriched with $CaCl_2(H_2O)_2$ solution with a molar concentration of 0.15 mM, pepsin (Sigma Aldrich) with an activity of 2000 U/ml. Subsequently, the oral digest was mixed with 8.1 ml SGF. The pH was then adjusted to 3 (using pH meter WTW Series, indoLab) with HCl (VWR) and the whole mixture was made up to 1:1 with distilled water. The samples were incubated at 37 °C in an incubator (Schoeller) with constant shaking for 2 hours.

In the intestinal phase of digestion, it was digested and mixed with 11 ml SIF. The simulated intestinal juice was enriched with bile (Sigma Aldrich) at a concentration of 10 mM (final concentration after mixing with the digestate), $CaCl_2(H_2O)_2$ solution at a molar concentration of 0.6 mM, pancreatin suspension (Sigma Aldrich), which was diluted to give a trypsin activity of 100 U/ml in the final mixture. Subsequently, the pH was adjusted to 7 with NaOH (Lachner) and the mixture was made up to a 1:1 ratio with distilled water. Samples were incubated at 37 °C in a heater incubator with constant shaking for two hours.

After completion of the intestinal part, the digestate samples were immediately placed in a freezer at -80°C to stop digestion.

4.3. Cytotoxicity test

Cell viability was determined using the (MTT) 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (Sigma-Aldrich s.r.o., USA) cytotoxicity test, which was originally established by Mosmann (1983) and modified. Two cell lines of human colorectal adenocarcinoma, HT-29 (ATCC HTB-38) and Caco-2 (ATCC HTB-39) were seeded in 96-well plates at a density of 2.5×10^3. After 24 hours, cells were treated with two-fold serially diluted samples (2,4 to 20 000 μg/ml of digestate) for 72 hours. Then, MTT reagent (1mg/ml) in Eagle's minimum essential medium (EMEM) was added to each well and incubated for another 2 hours at 37 °C with 5% CO_2. The medium containing MTT was removed, and the intracellular formazan product was dissolved in 100 μl of dimethylsulfoxide. The absorbance at 555 nm was measured using a Tecan Infinite M200 spectrometer (Tecan Group, Männedorf, Switzerland), and the percentage of viability (IC_{50} and IC_{20}) was computed in comparison to the untreated control.

Statistical analysis was carried out using MagellanTM software (Tecan Group, Männedorf, Switzerland) and Microsoft M365 (Microsoft, Redmond, WA, USA) on data from three separate trials.

4.4. Adhesion properties of *Lactobacillus* species on cell culture model

Adhesion was determined using the modified method reported by Krausova et al. (2019). The co-culture of human colorectal carcinoma cell lines HT29 (ATCC HTB-38) and Caco-2 (ATCC HTB-39) was used. This co-culture cell line was grown in EMEM supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 1% penicillin/streptomycin (10,000 units of penicillin and 100 mg of streptomycin, Sigma-Aldrich), and 1% sodium bicarbonate (Sigma-Aldrich) in a humid atmosphere at 37 °C and 5% CO_2. Before the experiment, the co-culture cell line (a mixture of HT29 and Caco- 2) was seeded in a 96-well fluorescent white microtiter plate with a density of 1×10^5 cells/well. The cells were then incubated in EMEM until a minimum of 95% confluence was achieved (usually about 72 h). Prior to the adhesion experiment, all evaluated human bacterial species (ATCC, USA) were grown for 24 hours in standard culture broth. The bacterial suspension was rinsed twice with phosphate buffer solution (PBS). The bacterial suspension was fluorescently marked by adding 25 μg/ml of fluorescein (Thermo Fisher Scientific, USA) dissolved in 1ml sodium bicarbonate and incubated for 1 hour at 37°C in the dark. The bacterial suspension was washed twice in PBS to achieve a final concentration of 1×10^7 CFU (colony-forming units). The old medium was removed from the microtiter plate, and 90 μL of the new medium without additives and 10 μl bacterial suspension with testing samples (in concentrations of 256, 128 and 64 μg/ml) were added. The plates were incubated for 1.5 hours at 37°C and 5% CO_2 in the dark. All wells, including those with and without tasting samples, were rinsed twice with 100 μL PBS before adding another 100 μl PBS. Fluorescence was detected at 478/510 nm using the TECAN Infinite M200 reader (Tecan Infinite M200 reader). All experiments were performed in triplicate. The percentage of adhesion was calculated as follows:

$$X\ (\%) = (RFU\ sample / RFU\ control) \times 100$$

where X (%) is % of fluorescence in the well; RFU sample = well fluorescence in relative fluorescence units, with tasting sample. RFU control = well fluorescence in relative fluorescence units, without tasting sample.

4.4.1. Statistical evaluation

The obtained results are presented as the mean and standard deviation. A two-way analysis of variance ANOVA in Statistics was used to process the resulting data, with a materiality level of $\alpha = 0.05$.

5. Results

The results of viability of HT29 and Caco-2 are shown below (Table 2). In the case of QS extract with Caco-2 cell line, cytotoxicity was determined as follows: IC_{20}= 172 μg/ml and IC_{50}= 419 μg/ml. No cytotoxic effect of the QS extract was observed in the HT29 cell line. In the case of QY extract with the Caco-2 cell line, cytotoxicity was determined as follows: IC_{50}= 314 μg/ml and for the IC_{20} no cytotoxicity was determined. No cytotoxic effect of QY extract was observed in HT29 cell line.

The IC_{20} of QS extract for Caco-2 was then used to determine proper concentrations for adhesion experiment, where IC_{20} and its double dose and half dose were used (300, 150, 75 μg/ml). The toxic effect of QS extract in the highest concentration on cell monolayer formation was demonstrated by microscopic photography. An intact monolayer without exposure to QS extract is seen (Figure 10) and a damaged monolayer exposed to 300 μg/ml QS extract (Figure 11).

Figure 10. Intact cell monolayer without presence of QS and QY extract

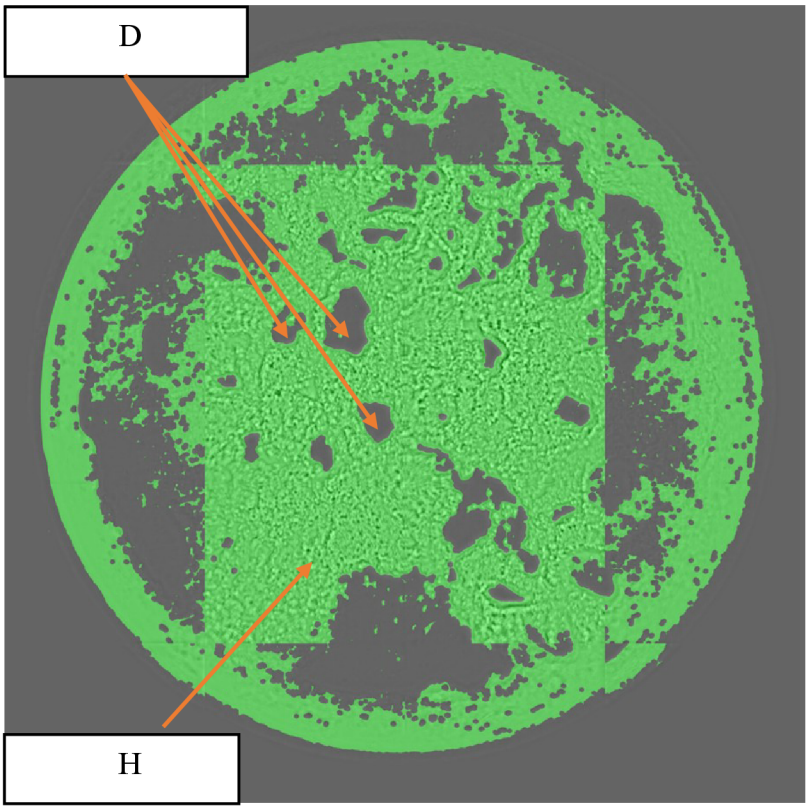

Figure 11. Monolayer after exposure to QS extract at 300 µg/ml concentration:

D = areas of damaged cells; **H** = healthy cells

Table 2. Toxicity of QS and QY extracts to intestinal cell lines Caco-2 and HT29

Samples	Caco-2 digestate ± SD (µg/ml)		HT29 digestate ± SD (µg/ml)	
	IC_{50}	IC_{20}	IC_{50}	IC_{20}
Q. saponaria	419 ± 30	172 ± 44	>20	n.d.
Q. saponaria, Y. schidigera	304 ± 34	n.d.	>20	n.d.

n.d. – not defined

After evaluating cytotoxicity and determining the proper concentration of extracts, an adhesion experiment was performed. The effect of QS (Figure 12) and QY (Figure 13) extracts on the adhesion of selected species of LAC: *Lactobacillus brevis, L. gasseri* and *Lacticaseibacillus rhamnosus,* was evaluated. Three different concentrations of QS and QY extracts were tested on the co-culture of colorectal carcinoma cell lines (Caco-2 and HT29). The results were expressed in % as mean and ± standard deviation and were compared with the control (100% adhesion).

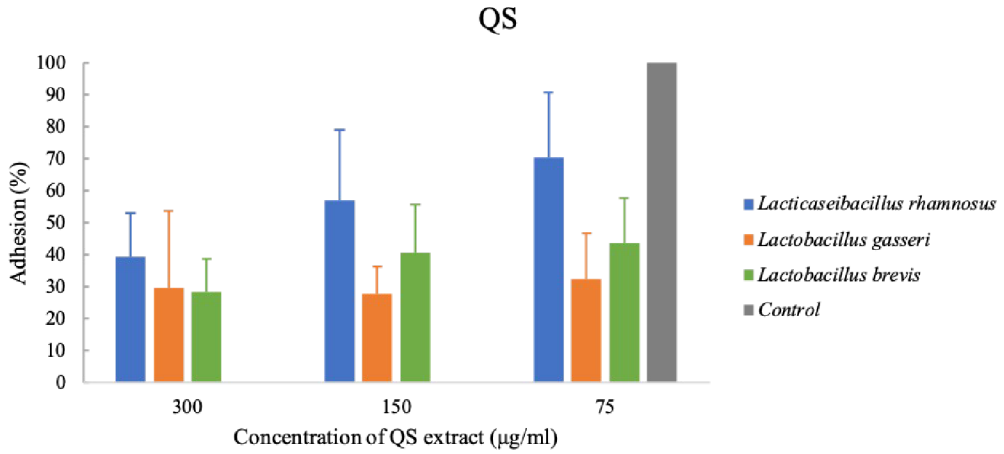

Figure 12. Adhesion of selected LAC species in the presence of QS extract to a monolayer of co-culture of HT29 and Caco-2 cells.

For *Lacticaseibacillus rhamnosus*, adhesion was reduced the least, and worsened with increasing concentration of the tested QS extract. The second highest reduction in adhesion was found for *L. brevis* with a lower dependence on the tested QS sample concentration. In the case of *L. gasseri*, the reduction in adhesion was most significant even at the lowest concentration and it appears that for this organism, concentration of QS sample had no effect on the changes in adhesion (Table 3).

In the case of QY extract it was found that adhesion was reduced in all but one case (Figure 13).

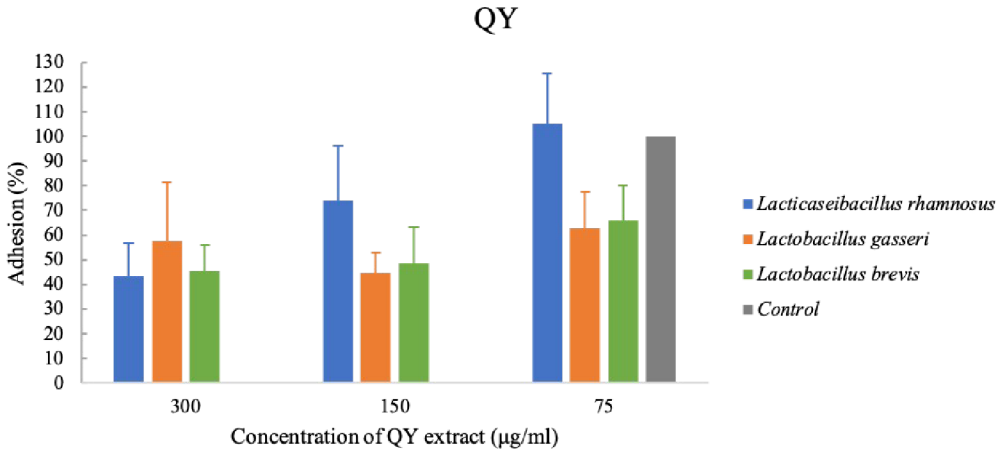

Figure 13. Adhesion of selected LAC species in the presence of QY extract to a monolayer of co-culture of HT29 and Caco-2 cells.

In the case of *Lacticaseibacillus rhamnosus* at the lowest concentration (0.075) of QY sample, adhesion was positively affected. Also, for this species, compared to the other LAC species tested, adhesion was reduced the least at the second highest sample concentration (0.15). In the case of *L. brevis*, the results showed that the ability of this LAC species to adhere decreases with increasing sample concentration. At the highest QY concentration, adhesion was reduced the least in *L. gasseri*, compared to the other LAC (Table 3).

Table 3. Effect of QS and QY extract on adhesion of LAC to intestinal epithelial cells (Co -culture of HT29 and Caco-2 cells)

Samples	Concentration of digestate in (µg/ml)	*Lacticaseibacillus rhamnosus*	*Lactobacillus gasseri*	*Lactobacillus brevis*
		mean ± SD (%)		
Q. saponaria	300	39.28 ± 13.64*	29.66 ± 24.02*	28.49 ± 10.29*
	150	56.99 ± 22.09*	27.87 ± 8.50*	40.77 ± 15.10*
	75	70.49 ± 20.06*	32.40 ± 14.48*	43.66 ± 14.07*
Q. saponaria, Y. schidigera	300	43.31 ± 17.67*	57.51 ± 11.23*	45.73 ± 11.60*
	150	74.22 ± 20.34*	44.55 ± 19.43*	48.38 ± 18.79*
	75	105.34 ± 19.88	63.01 ± 24.77*	65.88 ± 17.96*
Control (%)	0	100	100	100

Asterisks show the significance of the mean values of three measurements compared to the control *p<0.05

In all but one case, lactobacillus adhesion was statistically significantly affected compared to the control. In all statistically significant cases, the ability to adhere was reduced (Figure 14, 15). Only in the case of *Lacticaseibacillus rhamnosus* was adhesion positively affected in the presence of the QY sample, but the effect was not statistically significant (Figure 16).

In the case of *L. brevis* (Figure 14), the reduction in adhesion of individual extracts was not dependent on the concentration used. However, there was a statistically significant difference in adhesion between QY extract at the lowest concentration and QS extract at the highest concentration.

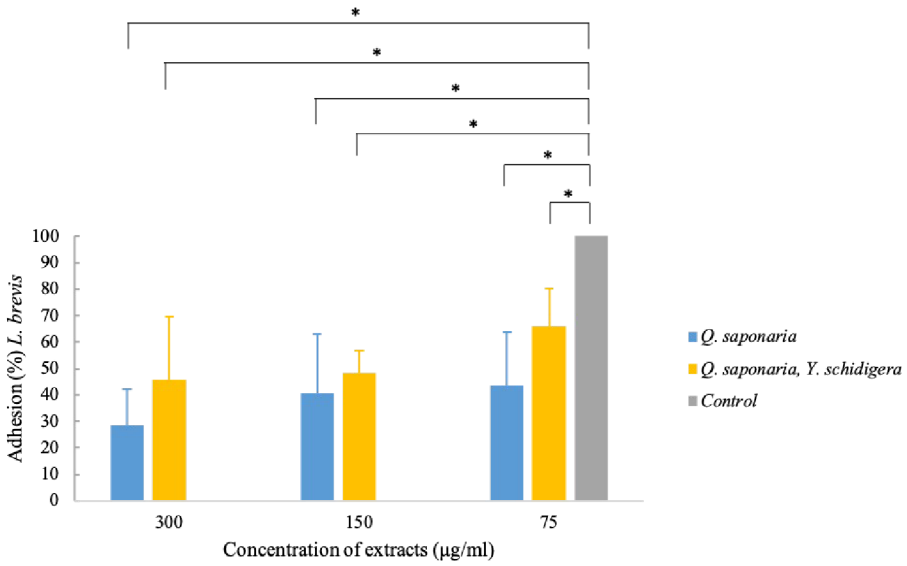

Figure 14. Ability of *L. brevis* to adhere in the presence of QS and QY extracts compared to control. *p<0.05

In the case of *L. gasseri* (Figure 15), the middle concentration of QY extract statistically significantly impaired adhesion compared to the lowest dose, but this is likely an outlier value as adhesion of this species was not different at the lowest concentration compared to the highest. Also, in the case of *L. gasseri*, there was a statistically significant difference in adhesion reduction between QS and QY samples at the highest concentration, with the QY sample reducing adhesion less.

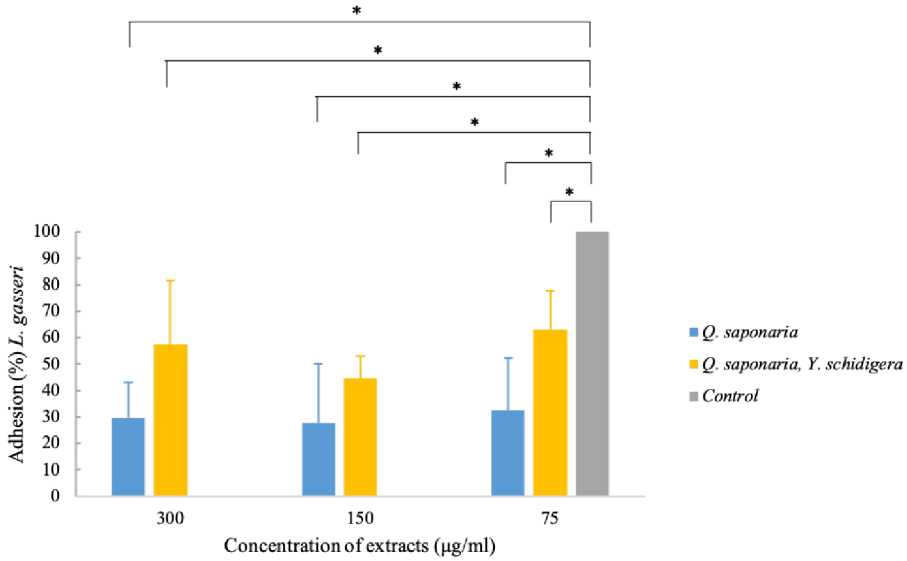

Figure 15. Ability of *L. gasseri* to adhere in the presence of QS and QY extracts compared to control. *p<0.05

Lacticaseibacillus rhamnosus (Figure 16) was the most sensitive of the LAC species tested to the applied doses of saponins. For the QY extract, adhesion decreased with increasing concentration. A statistically significant difference in adhesion reduction was, for example, demonstrated in *Lacticaseibacillus rhamnosus* between the lowest and the highest applied concentration of QY extract. A statistical difference in the ability of *Lacticaseibacillus rhamnosus* to adhere was also demonstrated for the lowest concentrations of QY and QS, with the QY sample even having a positive effect on adhesion.

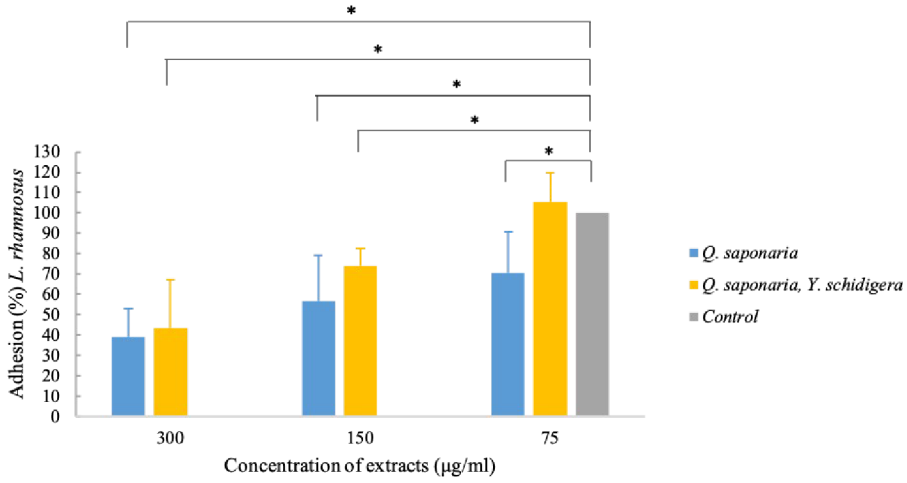

Figure 16. Ability of *L. rhamnosus* to adhere in the presence of QS and QY extracts compared to control. *p<0.05

Our findings revealed that not only the lactobacilli species, but also the tested extracts, differed significantly. The QY sample reduced adherence less than the QS extract.Detailed comparisons of the tested extracts at different concentrations on individual lactobacilli species, as well as interspecies differences, are expressed in detail in Appendix 1, including statistical significance.

6. Discussion

The aim of this study was to determine the influence of extracts of *Quillaja saponaria* and the combination of extracts of *Quillaja saponaria* and *Yucca schidigera* on *in vitro* adhesion of selected LAC species. Due to the potential toxicity of saponins, a cytotoxicity assay was used to determine appropriate concentrations for the adhesion experiment.

For detemination of adhesion, individual LAC species *(L. rhamnosus, L. gasseri, L. brevis*) were exposed to sellected concentrations of QS and QY saponin extracts and tested for their ability to adhere to the human intestinal epithelial carcinoma co-culture of Caco-2 and HT29 cell lines. Choosing the correct extract concentration for the adhesion experiment using the cytotoxicity assay was crucial, as it is known from the literature that saponin extracts can be toxic at higher concentrations (Marciani 2015; E. Walkowicz et al. 2016).

Saponins from QS and YS plants have already been widely tested for antimicrobial, antiviral, antiparasitic, anticancer activities and many other (Killeen et al. 1998; Wisløff et al. 2008; Tam & Roner 2011; Serrano 2013; Onbaşilar et al. 2014; Bafundo et al. 2020, n.d.). Since these saponin extracts are commercially sold preparations, their composition may vary slightly, and thus the biological effect of the extracts may also differ. Differences in saponin extracts have been demonstrated, for example, by Sen et al. (1998) on the antimicrobial activity of saponins against *E. coli*.

Most *in vitro* studies focusing on the biological activity of saponins have tested these substances without simulating passage through the gastrointestinal tract. However, as reported, for example, by Chen et al (2022), sapogenins, mainly phytolaccagenin (PA), hederagenin (HD), and oleanolic acid from quinoa plant are released during the acid hydrolysis process. However, he adds that during gastrointestinal digestion there is no release of sapogenins from quinoa and there is a reduction in antioxidant activity.

In our cytotoxicity experiment, QS and QY extracts were tested separately on two epithelial tumor cell lines, HT29 and Caco-2. The results showed that the extracts

had toxic effect on Caco-2 cells with IC_{50} = 419 μg/ml for QS extract and IC_{50} = 304 μg/ml for QY extract. In the case of the HT29 cell line, no toxicity of the saponin extracts at the tested concentrations was detected. In the contrary saponin mixtures were also tested in the work of Kozińska et al. (2021) where the mixtures were tested on the MRC-5 lung fibroblast cell line. It was found that there were differences in the ability to reduce viability of cells at concentrations around 50 μg/ml depending on the extract. A significant cytotoxic effect was also observed conducted by Tavares et al. (2021) in the hepatic hepatocyte cell line HepG2 where extract from *Sapindus saponaria* was tested. The IC_{50} value for saponins from this species was determined by the method using tetrazolium salt WST-1 at 180.4 ± 9.81 μg/ml. The fact that the two studies mentioned above determined the cytotoxicity of saponins to be significantly higher than our test may be influenced by the fact that the saponin samples used in these studies had not been previously digested in an *in vitro* digestion model. Our extracts were digested in an *in vitro* digestion model prior to the cytotoxicity experiment to obtain the most realistic results. If simulated *in vitro* digestion is not performed prior to testing the biological activity of saponins on intestinal cell lines, the results may be different and less realistic. In the study conducted by Navarro del Hierro et al. (2018) suggest that the processes during digestion of saponins are largely related to their biological activity, as saponins are poorly absorbed and remain in the intestinal tract for long periods of time. According to this study, digestion modulates the bioavailability and bioaccessibility of saponins and influences their relationship with the resident microbiota. Thus, exposure of saponins to gastric solutions in our expriment may have altered the biological activity of these saponins, leading to their lower toxicity to gut bacteria.

The different cytotoxic effects of the tested saponin extracts on the Caco-2 and HT29 cell lines may be caused by their physiological differences. These two cell lines have different mucin production profiles. Cell line HT29 represents goblet cells and produces mucin, while the Caco-2 cell line does not produce mucin and represents enterocytes (Laparra & Sanz 2009). The absence of cytotoxic effect of saponin extracts against HT29 cells might have resulted from mucin production by HT29 cells because it serves as a protective layer that protects the cells from adverse conditions.

The main hypothesis of this study was that extracts from QS and QY would affect lactobacilli adhesion. We confirmed this hypothesis as adhesion was significantly

affected in all but one case. In all statistically significant cases, lactobacilli adhesion was reduced at all tested concentrations. The opposite phenomenon was observed by Luo et al. (2023), according to his results saponins isolated from *Polygonatum sibiricum* had no negative effects on the growth of selected probiotic bacteria after 24h incubation and their viability increased, however, the effects varied among species. The ability of probiotic bacteria to adhere to intestinal epithelial cells is health-promoting and positively affects the binding potential of pathogens. However, this ability may be influenced by the cell line used in *in vitro* testing as well as the mechanism of interaction between LAC and the cell surface. This is mainly related to the production (non-production) of mucus (Gagnon et al. 2013).

In our test the least affected LAC species was *L. rhamnosus*, which showed the lowest reduction of adhesion compared to the other LAC species tested in presence of both QS and QY samples. In the case of *L. rhamnosus* adhesion was even positively affected at the lowest concentration of the QY sample. This finding suggests that the concentration and a particular species of lactobacilli plays a role in the ability to resist negative effects of these saponin extracts. The fact that *L. rhamnosus* showed greater resistance than the other LAC species tested may result from the fact that this species is known for its ability to form biofilms on abiotic surfaces and its very good adherence to epithelial cells under *in vitro* conditions (Lebeer et al. 2007; Martín et al. 2019). The biofilms of LAC are their own extracellular matrix that protects them from adverse environmental conditions. In a study conducted by Lebeer et al. (2007), it was found that *L. rhamnosus* is able to form biofilms even under *in vitro* conditions, which is not the case for all LAC species. However, the fact that *L. rhamnosus* is more resistant remains to be verified in further *in vitro* studies. If *L. rhamnosus* is shown to be more resistant in other tests, this LAC species could serve as a probiotic and be added, to livestock feed containing saponins.

The results of our experiment showed that not only the species of lactobacilli but also the tested extracts themselves showed noticeable differences. The QY sample reduced adhesion less compared to the QS extract.Since QS was a pure extract of *Quillaja saponaria* and QY was a mixture of *Quillaja saponaria* and *Yucca schidigera* extracts, our results confirmed the results previously published by Hassan et al. (2010) that *Yucca* has different biological activity than *Quillaja*. This fact may be due to the

different chemical composition of the two extracts. The saponins from *Quillaja* and the saponins from *Yucca* differ in their chemical structure. In the case of *Quillaja*, the saponins consist of triterpene structures (Guo & Kenne 2000), whereas in the *Yucca*, they are steroidal saponins (Joanne et al. 2007). Also in the study conducted by Dai et al. (2023) which compared the effect of QS and YS extracts on broiler growth performance, immunity, antioxidant capacity and intestinal flora, it was found that YS extract has greater benefits in improving feed gain ratio, immunity and intestinal morphology and reducing inflammatory factors in broiler serum compared to QS extract. These positive effects of YS can be related to lactobacilli adhesion and colonization of the gut.

Since the adhesion of lactobacilli was significantly reduced, we can also assume that saponin extracts could reduce adhesion of undesirable pathogens such as *Escherichia coli* to intestinal epithelial cells. Moreover, it is well known that positive microorganisms colonize the gastrointestinal tract slowly than pathogenic microorganisms, but much more efficiently (Canny & McCormick 2008). Pathogens are more aggressive in colonization but are more sudden to changes in the environment. Saponins could, therefore, be used to eliminate pathogens in the gut by preventing them from adhering to the intestinal mucosa. This process would prevent pathogens from effectively colonizing the gut and potentially causing clinical problems. This hypothesis has to be verified in further research.

These findings offer a rough approximation of the behaviour of these LAC species in the presence of the saponin extracts in the gut. However, it is challenging to extrapolate *in vitro* experiment results about bacterial adhesion to epithelial cell lines to the human or animal gastrointestinal tract epithel, where bacterial adhesion is influenced by host gastrointestinal defence mechanisms, mucosa, peristaltic flow, and competition with resident microbiota (Lebeer et al. 2008).

7. Conclusions

To the best of our knowledge, this is the first time that saponin extracts of QS and QY were first digested using an *in vitro* digestion model and then used for *in vitro* adhesion assays. Our results showed different adhesion ability of the used LAC species in the presence of the tested saponin extracts. The ability of selected lactobacilli species (*L. rhamnosus, L. gasseri, L. brevis)* to adhere to colorectal carcinoma co-culture of cell lines HT29 and Caco-2 in the presence of QS and QY saponin extracts was affected in most cases. In all statistically significant cases, the ability of these lactobacilli to adhere to the epithelial cell line was reduced. Only one case showed an increase in adhesion. The adhesion of *L. rhamnosus* was increased at the lowest concentration of the QY extract. Overall, *L. rhamnosus* showed higher resistance to the tested saponin extracts than the other LAC species. In the comparison of QS and QY saponin extracts, we concluded that the sample containing *Yucca schidigera* extract in addition negatively affected lactobacilli adhesion less than the pure saponin extract of *Quillaja saponaria*. Further research is needed on the effect of saponin-containing extracts on both positive and potentially pathogenic bacteria. Effects *in vitro* have to be verified also in *in vivo* models to demonstrate the real effects in gastrointestinal tract.

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Appendices

List of the Appendices:

Appendix 1. Statistically significant differences in adhesion depending on the concentration of QS and QY samples used and lactobacilli species

	300 :*Lacticaseibacillus rhamnosus* QS	300:*Lactobacillus gasseri* QS	300 :*Lactobacillus brevis* QS
300: *Lactobacillus gasseri* QS	**		ns
300: *Lactobacillus brevis* QS	ns	ns	
300: *Lacticaseibacillus rhamnosus* QY	*	****	****
300: *Lactobacillus gasseri* QY	ns	*	ns
300: *Lactobacillus brevis* QY	ns	*	ns
150:*Lacticaseibacillus rhamnosus* QS	ns	**	ns
150:*Lactobacillus gasseri* QS	ns	ns	ns
150:*Lactobacillus brevis* QS	ns	ns	ns
150:*Lacticaseibacillus rhamnosus* QY	***	****	****
105:*Lactobacillus gasseri* QY	ns	**	ns
150:*Lactobacillus brevis* QY	ns	**	ns
75:*Lacticaseibacillus rhamnosus* QS	***	****	****
75:*Lactobacillus gasseri* QS	ns	***	ns
75:*Lactobacillus brevis* QS	ns	*	***
75:*Lacticaseibacillus rhamnosus* QY	****	****	****
75:*Lactobacillus gasseri* QY	ns	****	***
75:*Lactobacillus brevis* QY	ns	****	***

	300 :*Lacticaseibacillus rhamnosus* QY	300:*Lactobacillus gasseri* QY	300 :*Lactobacillus brevis* QY
300:*Lactobacillus gasseri* QY	ns		
300:*Lactobacillus brevis* QY	*	ns	
150:*Lacticaseibacillus rhamnosus* QS	ns	ns	ns
150:*Lactobacillus gasseri* QS	***	ns	ns
150:*Lactobacillus brevis* QS	**	ns	ns
150:*Lacticaseibacillus rhamnosus* QY	ns	**	**
150:*Lactobacillus gasseri* QY	ns	ns	ns
150:*Lactobacillus brevis* QY	ns	ns	ns
75:*Lacticaseibacillus rhamnosus* QS	ns	ns	ns
75:*Lactobacillus gasseri* QS	ns	ns	ns
75:*Lactobacillus brevis* QS	ns	ns	ns
75:*Lacticaseibacillus rhamnosus* QY	***	****	****
75:*Lactobacillus gasseri* QY	ns	***	ns
75:*Lactobacillus brevis* QY	ns	ns	***

	150:*Lacticaseibacillus rhamnosus* QS	150:*Lactobacillus gasseri* QS	150:*Lactobacillus brevis* QS
150:*Lactobacillus gasseri* QS	**		
150:*Lactobacillus brevis* QS	ns	ns	
150:*Lacticaseibacillus rhamnosus* QY	*	****	****
150:*Lactobacillus gasseri* QY	ns	*	ns
150:*Lactobacillus brevis* QY	ns	*	ns
75:*Lacticaseibacillus rhamnosus* QS	ns	****	**
75:*Lactobacillus gasseri* QS	ns	ns	ns
75:*Lactobacillus brevis* QS	ns	ns	ns
75:*Lacticaseibacillus rhamnosus* QY	***	****	****
75:*Lactobacillus gasseri* QY	ns	***	*
75:*Lactobacillus brevis* QY	ns	***	*

	150:*Lacticaseibacillus rhamnosus* QY	150:*Lactobacillus gasseri* QY	150:*Lactobacillus brevis* QY
150:*Lactobacillus gasseri* QY	ns		
150:*Lactobacillus brevis* QY	*	ns	
75:*Lacticaseibacillus rhamnosus* QS	ns	ns	ns
75:*Lactobacillus gasseri* QS	***	ns	ns
75:*Lactobacillus brevis* QS	*	ns	ns
75:*Lacticaseibacillus rhamnosus* QY	ns	***	***
75:*Lactobacillus gasseri* QY	ns	ns	ns
75:*Lactobacillus brevis* QY	ns	ns	ns

	75:*Lacticaseibacillus rhamnosus* QS	75:*Lactobacillus gasseri* QS	75:*Lactobacillus brevis* QS
75:*Lactobacillus gasseri* QS	**		
75:*Lactobacillus brevis* QS	ns	ns	
75:*Lacticaseibacillus rhamnosus* QY	*	****	****
75:*Lactobacillus gasseri* QY	ns	*	ns
75:*Lactobacillus brevis* QY	ns	*	ns

	75:*Lacticaseibacillus rhamnosus* QY	75:*Lactobacillus gasseri* QY	
0.075:*Lactobacillus gasseri* QY	ns		
0.075:*Lactobacillus brevis* QY	*	ns	

Note: ****significant at 0.000 level, ***significant at 0.001 level, ** significant at 0.01 level, * significant at 0.05 level, ns=not significant. Concentartions of digestate: 75, 150, 300 μg/ml