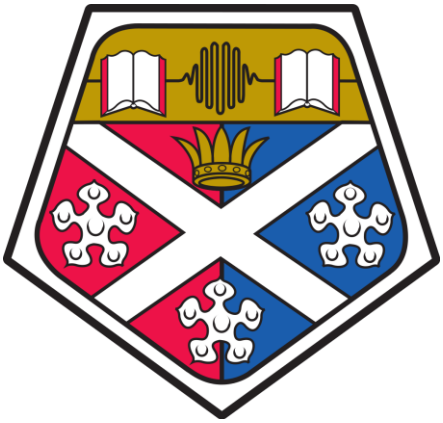

University of Strathclyde

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Novel Camel Milk-Derived Proteins and their Application in the Management of Acne Vulgaris

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Thesis presented in fulfillment of the requirements for

the degree of Doctor of Philosophy

2019

Declaration

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed:

Date:

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Dedication

To,

My lovely Mother

My supportive and caring beloved husband Ammar Abu Tarboush

My Lovely Children,

Batool, Layan, Aseel, and Ahmad

To,

The soul of my mentor, father, friend, who held my hand in the hardest time of my journey, and the one whom I would never came this far without him

Samih Darwazah,

(RIP)

Acknowledgment

{اقْرَأْ وَرَبُّكَ الْأَكْرَمُ الَّذِي عَلَّمَ بِالْقَلَمِ عَلَّمَ الْإِنسَانَ مَا لَمْ يَعْلَمْ}

العلق: 3 - 5

I would like to express my deepest appreciation and gratitude to my academic Supervisors, Prof. Mohammad Zourob and Dr. Ibrahim Khadra, and for their valuable guidance, advice, and support, throughout the course of my work.

I also extend my appreciation and gratitude to Prof. Khaled AlKattan and Alfaisal University.

My thanks to the members of the examination committee for devoting their valuable time to read and evaluate this work.

My supportive team at MonoJo:

Prof. Fahmi Abu Al-Rub, Rasha Hiary, Majd Hijjawi, Mohammad Alahmad, Sameer Nasrallah, Mohammad Malkawi, Dr. Luay Abu-Qatouseh, and Dr. Khaled Al-Qaoud,

I also would like to thank anyone who helped me in anything during this PhD journey,

I appreciate any support given no matter…

Patents and Publications

Patents

1) Camel Milk Based Pharmaceutical Composition - Granted in the UK 2507641

2) Camel Milk-Based Topical Pharmaceutical Composition - Granted in the USA 2014/0072648

Publications

Abu-Qatouseh L., Mallah E., Issa H., Sabri I., Shihab P. "Antimicrobial and Anti-inflammatory Activity of Camel Milk Derived Immune Proteins and Peptides against *Propionibacterium acnes*", International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS), January 2019, 8(1), page 163

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List of Abbreviations

Abbreviation	Meaning
AMPs	Antimicrobial Peptides
APS	Ammonium Per Sulfate
BSA	Bovine Serum Albumin
CFU	Colony Forming Unit
ELISA	Enzyme-Linked Immunosorbent Assay
HRP	Horse Radish Peroxidase
ID	Inhibitory Dose
Ig	Immunoglobulin
IL	Interleukin
kDa	Kilo Dalton
LPS	Lipopolysaccharide
MHA	Mueller Hinton Agar
NCTC	National Collection of Type Cultures
OD	Optical Density
OMPs	Outer Membrane Proteins
P. acnes	*Propionibacterium acnes*
PBS	Phosphate Buffered Saline
PGRP	Peptidoglycan Recognition Proteins
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TEMED	Tetramethylethylenediamine

THP-1	Human monocytes derived from Acute Monocytic Leukemia (American Type Culture Collection (ATCC))
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor

Abstract

Acne vulgaris, caused by the bacterium *P. acnes,* is the most common cutaneous disorder in adolescents. Acne is not a life-threatening condition, however, it has significant psychological and physical effects on patients. *P. acnes* is capable of causing remarkable inflammatory responses. Topical therapy is advisable in acne treatment in mild to moderate acne conditions. Recently, available treatments for acne are mostly based on antibiotics and retinoids. The use of antibiotics has the limitations of developing bacterial resistance, while retinoids are highly teratogenic. The bacterial resistance and teratogenicity of isotretinoin led scientists to search for more potent and safer therapeutic options. Camel milk has been used in the preparation of pharmaceutical and cosmetic compositions such as anti-acne products. It was found that camel milk composition is closer to the human milk. Passive immunity is provided to newborns by Immunoglobulins present in colostrum until its own immune system matures. The concentration in colostrum of specific antibodies against pathogens can be raised by immunizing a mammal with these pathogens or their antigens. Immunized milk products are preparations made of such hyper-immune antibodies enriched from it. Camel milk and milk-derived products have a growing potential therapeutic value. The objective of this research is to evaluate the antimicrobial and anti-inflammatory effects of immunized camel milk whey and its components against *P. acnes* and to develop a formula that harnesses

all its beneficial properties. *P. acne*s was heat-killed in culture to obtain their outer membrane proteins (OMPs). The OMPs were mapped and quantified using SDS-PAGE and Bradford Assay, respectively. OMPs were used to prepare a vaccination injected into female lactating camels in timely manner. The immunized camel milk was collected then pasteurized, delipidized, de-caseinated, and freeze-dried to obtain the whey into powder form. The anti-*P.acnes* antibodies in the immunized camel whey were separated using Protein G and Protein A Chromatography and quantified by SDS-PAGE and ELISA, respectively. Then, the antimicrobial peptides (AMPs) including PGRPs and Lactoferrin were isolated by Heparin Affinity Chromatography, and mapped by SDS-PAGE. The antimicrobial and anti-inflammatory activity of the immunized camel whey and its constituents against *P. acnes* was evaluated *in vitro. In vivo* activity was also evaluated using rabbit ear model. After the activity of the immunized camel whey was confirmed, four topical formulae were developed and their composition and preparation protocol were patented.

The findings of this research imply that the constituents of immunized camel milk whey (i.e. polyclonal anti-*P. acnes* antibodies, peptidoglycan recognition proteins (PGRPs), and lactoferrin) possess significant antimicrobial and anti-inflammatory activity against *P. acnes*, 90% and 70%, respectively. Topical formulae including; facial wash, serum, cream and gel enriched with 3% to 5% of immunized camel milk

whey were developed and their activity against *P. acnes* was evaluated. Immunized camel milk whey that has been developed in this research exerts significant antimicrobial and anti-inflammatory activity against *P. acnes* and the unique composition formulated has been patented. Furthermore, the stability of the topical cream was validated to show that the antibodies in the cream formula retained around 75% of their activity.

Chapter 1: Introduction

1.1 Introduction

Camels have played a significant socio-economic role in the lives of desert people in Asia and Africa with tremendous values for transportation and as a good food source (milk and meat). Camels belong to the family C*amelidae* and subordered as *Tylopoda*, *Artiodactyla* or cloven-footed animals. The family *Camelidae* includes two major tribes; the Old World *Camelina* tribe, which consists of the One-humped Dromedary (*Camelus dromedarius*) and the Two-humped Bactrian camel (*C. bactrianus*) species, and the second tribe is New World *Lamini* (Lamoids) consisting of its four species: guanaco (*Lama guanicoe*), llama (*L. glama*), alpaca (*L. pacos*) and vicuna (*L. vicugna* or *Vicugna vicugna*) (Novoa, 1989; Stanley *et al.*, 1994; Burger, 2016). The one-humped dromedary is native to the Arabian Desert while the two-humped Bactrian is found in Asia, and the lamoids are indigenous to South America (Daley *et al.*, 2005). The Food and Agriculture Organization estimates the total population of camels in the world to be 35 million heads (Fao, 2019).

1.2 Camel Milk

Milk production in camels takes longer time in arid zones and in harsh environmental regions than any other domestic livestock species, in spite of their modest food demand (Eissa *et al.*, 2010). The average daily yield of camel milk

production ranged from 3 to 10 liters in a lactation period of 12 to 18 months (Gizachew *et al.*, 2014). Camel milk is considered one of the main components of the human diet in many parts of the world. It contains all the essential nutrients found in cow milk (El-Agamy *et al.*, 1998) for growth and development such as proteins, minerals, carbohydrates, fatty acids, growth factors, immune modulators, etc. Various minerals, such as; Na, K, Ca, P, Mg, Fe, Zn, Cu and vitamins (A, E, C and B1) are present in camel milk (El-Khasmi *et al.*, 2001). In addition to numerus fatty acids, such as; butyric, caproic, caprylic, capric, lauric, myristic, myristoleic, palmitic, palmitoleic, stearic, oleic, linoleic and arachidonic acids (Narmuratova *et al.*, 2006). The fat content of camel milk ranges between 1.2% and 4.5% depending on the level of nutrition, stage of lactation, breed and the season, etc. Camel milk fat is characterized by a higher proportion of unsaturated fatty acids (Zhang *et al.*, 2005) and long-chain fatty acids (Konuspayeva *et al.*, 2008) compared to other species. Generally, Camel milk has opaque white color, faint sweetish odor and a sharp taste that is sometimes salty (Abbas *et al.*, 2013). It has a pH value of 6.2-6.5 and a density that ranges from 1.026-1.035 (Gul *et al.*, 2015). Generally, the average percentage of camel milk components is 3.4% protein, 3.5% fat, 4.4% lactose, 0.79% ash and 87% water (Al Haj and Al Kanhal, 2010).

1.2.1 Camel Milk Proteins

Based on their molecular characterization, camel milk proteins have unique properties contributed to the higher digestibility and lower allergy, which reflect their composition and structure compared to the proteins present in cow and human milk (El-Agamy *et al.*, 2009). Several factors attribute to the variations in the composition of camel milk such as breed, age, health status, nutritive conditions, seasonal stage of lactation, geographical origin, and analytical methods used (Khaskheli *et al.*, 2005; Konuspayeva *et al.*, 2008).

Milk proteins are heterogeneous group of biomolecules that differ in structure and composition and properties (Gizachew *et al.*, 2014). Dromedary camel milk contains two main protein groups; casein complexes and whey protein fractions (Abbas *et al.*, 2013; Gul *et al.*, 2015). Casein (cheese protein), is more important and is higher in concentration in camel milk, while whey proteins are relatively low (Guo *et al.*, 2007). Casein proteins represent the majority of dromedary camel milk protein; that is 1.63%-2.76%, which translates to 52%-87% of total milk protein (Khaskheli *et al.*, 2005). Casein is composed of four main fractions; αs1 and αs2-caseins, ß-casein, and κ-casein (Barłowska *et al.*, 2007). The αs1-casein fraction is the predominant factor behind milk protein allergies and is present in humans, cows, buffaloes and camels (Zicarelli, 2004). However, camel milk has more digestibility and less

allergic reactions in children as αs1-casein hydrolyzes slowly compared to β-casein (El-Agamy *et al.*, 2009).

On the other hand, whey protein constitutes 0.63%-0.80% of the dromedary camel milk, which translates to 20%-25% of the total protein (Khaskheli *et al.*, 2005). Whey protein present in camel milk consists of: 0.718 ± 0.330 mg/ml immunoglobulins, 0.22 mg/ml lactoferrin, 0.12 mg/ml peptidoglycan recognition protein (PGRP), $5*10^{-3}$ mg/ml lysozyme, 2.23 ± 0.01 units/ml (activity) lactoperoxidase, 2.01 ± 0.02 mg/ml α-lactalbumin and 0.46 ± 0.01 mg/ml serum albumin (Kappeler *et al.*, 2003). Compared to cow and buffalo milk, camel whey contains a higher content of immuno-protective proteins such as PGRP (Peptidoglycan Recognition Protein), lysozyme, lactoferrin and immunoglobulins (El-Agmey *et al.*, 1992; El-Hatmi *et al.*, 2007). On the contrary, camel milk whey lacks β-lactoglobulin (Kappeler, 1998), which is a major serum protein that makes 50% of the total whey protein of bovine milk (Abbas *et al.*, 2013) that is reported to be the major source for allergy in children in addition to αs1-casein and αs2-casein (Lara-Villoslada *et al.*, 2005). The lack of β-lactoglobulin in milk has also been reported in other species, including rodents and humans (Hambling *et al.*, 1992) which makes camel milk the most suitable alternative to human milk that will reduce children's allergic reactions (Shabo *et al.*, 2005).

1.2.1.1 Peptidoglycan Recognition Protein (PGRP)

PGRP is a soluble and conserved pattern recognition protein of vertebrates and invertebrates that binds to bacterial peptidoglycan (an integral part of the cell walls of almost all bacterial types) with high affinity and plays a significant role in the host's innate immune system (Liu *et al.*, 2000; Kappeler *et al.*, 2004).

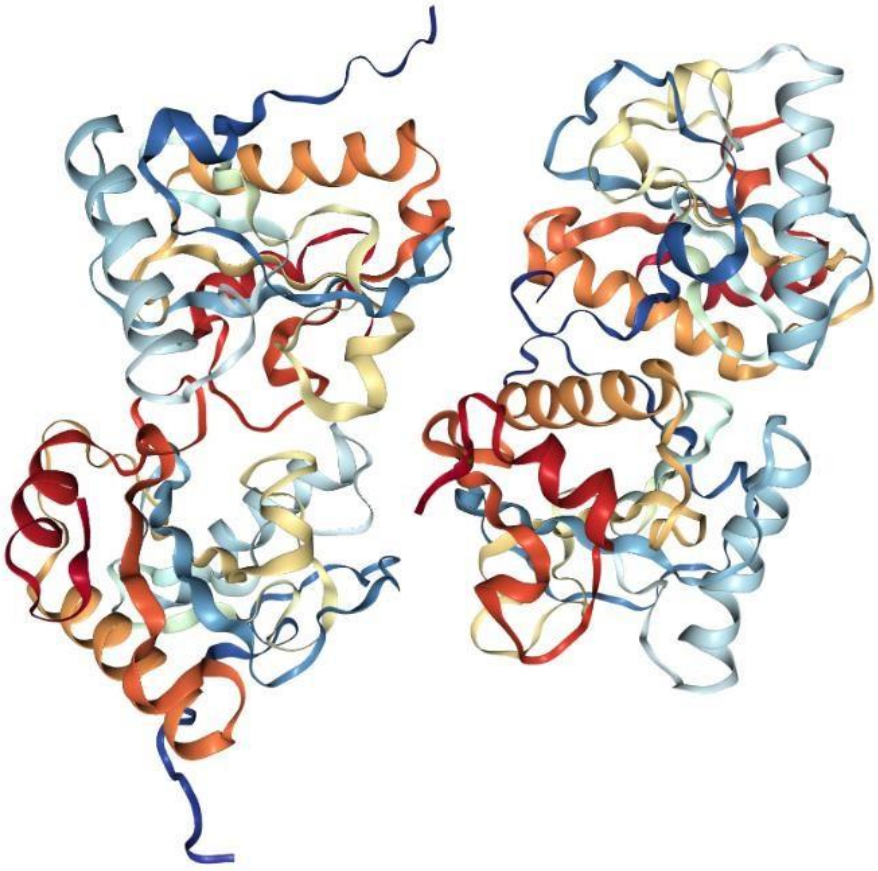


Figure 1.1: Crystal Structure of Cameline Peptidoglycan Recognition Protein at 2.8A Resolution (Adopted from the Protein Data Bank coded 2R90, www.rcsb.org/3d-view/2R90/1)
D. Sehnal, S. Bittrich, M. Deshpande, R. Svobodová, K. Berka, V. Bazgier, S. Velankar, S.K. Burley, J. Koča, A.S. Rose (2021) Mol* Viewer: modern web app for 3D visualization and analysis of large biomolecular structures. Nucleic Acids Research. doi: 10.1093/nar/gkab314)

Investigations have shown that members of this protein family recognize a range of microorganisms and their cell envelope constituents (Liu *et al.*, 2000; Kappeler *et al.*, 2004; Tydell *et al.*, 2006). Mammalian PGRPs have been identified in humans, rats, mice, cattle, camels, and pigs (Tydell *et al.*, 2006). Camel milk PGRP binds to lactic acid, lipopolysaccharide (LPS) and lipoteichoic acid (LTA) of gram-positive and pathogenic bacteria (Kappeler *et al.*, 2004; Sharma *et al.*, 2011). Previous studies have indicated the useful properties of camel PGRP as an antibiotic agent (Sharma *et al.*, 2011; Sharma *et al.*, 2013). The crystal structure of Cameline Peptidoglycan Recognition Protein at 2.8A Resolution is shown in Figure 1.1. The hydrolytic active site of PGRP-SA is difficult to pinpoint due to overlap of all residues involved in the binding pocket and other residues contributing to the small size of the pocket in PGRP-SA.

1.2.1.2 Lactoferrin

Lactoferrin is a mammalian cationic iron-binding glycoprotein that belongs to the transferrin family with its major function being iron transportation and/or storage. It is widely distributed in all biological fluids and is also expressed by immune cells, which release it under stimulation by pathogens (Habib *et al.*, 2013). Camel milk lactoferrin content is 0.22 $mg.ml^{-1}$, which is significantly higher than goat, sheep, buffalo and cow milk (Abbas *et al.*, 2013). Lactoferrin is considered one of the protective proteins in camel milk, its protective characteristics have been demonstrated in numerous studies in terms of antioxidative factors, antibacterial, antiviral, antifungal, among others. Lactoferrin has even been reported to inhibit the development of experimental cancer metastases in mice (Wang *et al.*, 2000; Wolf *et al.*, 2003) and tumor growth (Fujita *et al.*, 2004).

1.2.1.3 α-Lactalbumin

α-Lactalbumin is a major whey protein component in the milk of all mammalian species that belong to the lysozyme super-family (Nitta and Sugai, 1989). Camel α-lactalbumin was reported to have a molecular mass of 14.6 kDa and to comprise 123 residues, which is similar to bovine, human and goat milk α-lactalbumin (Beg *et al.*, 1986). The main biological function of this protein is to regulate lactose synthesis and transport vitamins and metabolites (Calderone *et al.*, 1996). The average

concentration of α-lactalbumin in camel colostrum and milk is 2.2 mg/ml (El-Hatmi *et al.*, 2007).

1.2.1.4 Lactoperoxidase

Lactoperoxidase is found in tears, saliva and milk. It was extracted and purified from bovine and camel milk with an approximate molecular weight of 88 and 78 kDa, respectively (Yoshida and Ye, 1991). Lactoperoxidase is a monomeric protein that contributes to the non-immune host defense system and is quite resistant to acidic and proteolytic digestion (Abbas *et al.*, 2013). It plays an important role in protecting the lactating mammary glands and the intestinal tract of newborn infants against pathogenic microorganisms (Reiter *et al.*, 1980; Leigh *et al.*, 1990). Its anti-tumor activity has also been reported (Stanislawski *et al.*, 1989).

1.2.1.5 Lysozyme

Lysozyme is a type of glycan hydrolases, which hydrolyzes the β-1,4-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in the peptidoglycan of bacterial cell walls (Jollès and Jollès, 1984). It plays an integral role as a non-specific immune factor and as an anti-inflammatory factor that is present in many mammalian fluids (e.g., blood, tears, milk) (Prager and Jolles, 1996; Ogundele, 1998). Camel lysozyme has been measured in both camel and bovine milk

and is reported to be 5*10-3 mg/ml compared to 0.37*10-3 mg/ml in bovine milk with a similar molecular weight of 14·4 KDa (Elagamy *et al.*, 1996).

1.3 The Immune System of the Camel

The camel has a unique immunological system that is different from all of all other mammals. Camel milk immunoglobulin G (IgG) consists of three main sub-classes, namely IgG1, IgG2, and IgG3; the two latter sub-classes are devoid of light chains, named heavy-chain antibodies (HCAbs), and have a molecular mass of about 100kDa (Hamers-Casterman *et al.*, 1993). Besides their reduced size, heavy-chain Igs have good solubility and stability (Muyldermans, 2001). Also, they display a higher level of specificity that can recognize regions that are less antigenic for conventional antibodies (Lauwereys *et al.*, 1998; Conrath *et al.*, 2005). Furthermore, heavy-chain Igs can be absorbed by the gut since their structure makes them very stable and able to resist the effect of acidic pH and gastric enzymes in the gut (Dumoulin *et al.*, 2002). Heavy-chain Igs act as true competitive inhibitors by penetrating the active sites of some enzymes (Lauwereys *et al.*, 1998). It has been suggested that the functional domain of the heavy chain antibodies, referred to as VH, interferes with several biological processes and may be a good candidate for human therapy (Holt *et al.*, 2003). In addition, camel antibodies have low immunogenicity, and can be easily humanized because of their high homology to the human antibodies (De Genst *et al.*, 2006).

1.4 Acne Vulgaris

1.4.1 Introduction

Acne Vulgaris is a common chronic inflammatory disease of the pilosebaceous unit, it is characterized by lesions that are distinctly categorized as non-inflammatory (open and closed comedones), or inflammatory (papules, pustules, and nodules). Inflamed lesions often precipitate scars after inflammation has cleared (Bhate & Williams, 2013). The disease affects mostly the sebaceous glands in the face, back, and trunk. The presence of the obligated anaerobic Gram positive bacterium *Propionibacterium acnes (P. acnes*) in the follicular and sebum canals is essentially involved in the development of inflammatory acne (Agrawal et al., 2004). *P. acnes* is capable of interacting with the components of the immune system and has the ability to bio-metabolize sebum fats into free fatty acids, leading to a remarkable inflammatory response presented with chemotaxis of neutrophils and induction of monocytes to produce various pro-inflammatory mediators including tumor necrosis factor (TNF) and Interleukin -8 (IL-8) (usFDA, 2000). Four main pathogenic mechanisms of Acne Vulgaris have been identified; seborrhea or increased production of sebum, hyperkeratinization of the follicles, colonization by *Propionibacterium acnes*, and inflammatory components. Although acne might occur at any age, it is most commonly encountered during adolescence (Rathi, 2011). Recently, a taxonomic reclassification was proposed in which *P. acnes* was renamed

Cutibacterium acnes to account for genomic adaptive changes and to differentiate it from other propionibacteria species (Platsidaki, E., & Dessinioti, C., 2018).

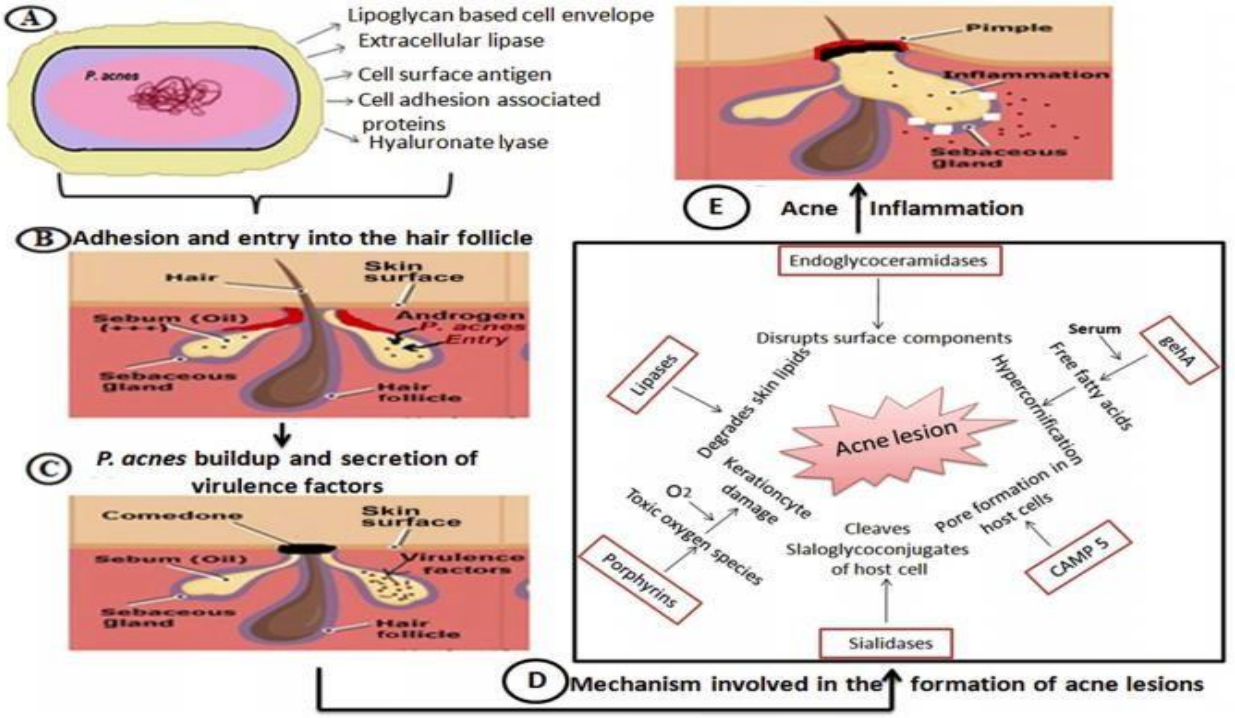


Figure 1.2: Mechanism of acne formation. Adopted from (Pathak, et al, 2015)
BipulKumar[1a]RajivPathak[1a]P. BertinMary[1]DikshaJha[1]KabirSardana[2]Hemant K.Gautam[1] 2015,from New insights into acne pathogenesis: Exploring the role of acne-associated microbial populations

1.4.2 Morphology

P. acnes is a gram positive anaerobic bacillus that characteristically produces propionic acid as a fermentative microbial product. *P. acnes* can be found in many human body parts; from the conjunctivae, oral cavity, and nares to the intestinal and respiratory tracts. It normally comprises around 50% of the human skin normal flora, but it can be pathogenic at times, resulting in a number of skin diseases, of which is

Acne Vulgaris (Behzadi, et al., 2010). Morphology of *P. acnes* is shown in below Figure 1.2.

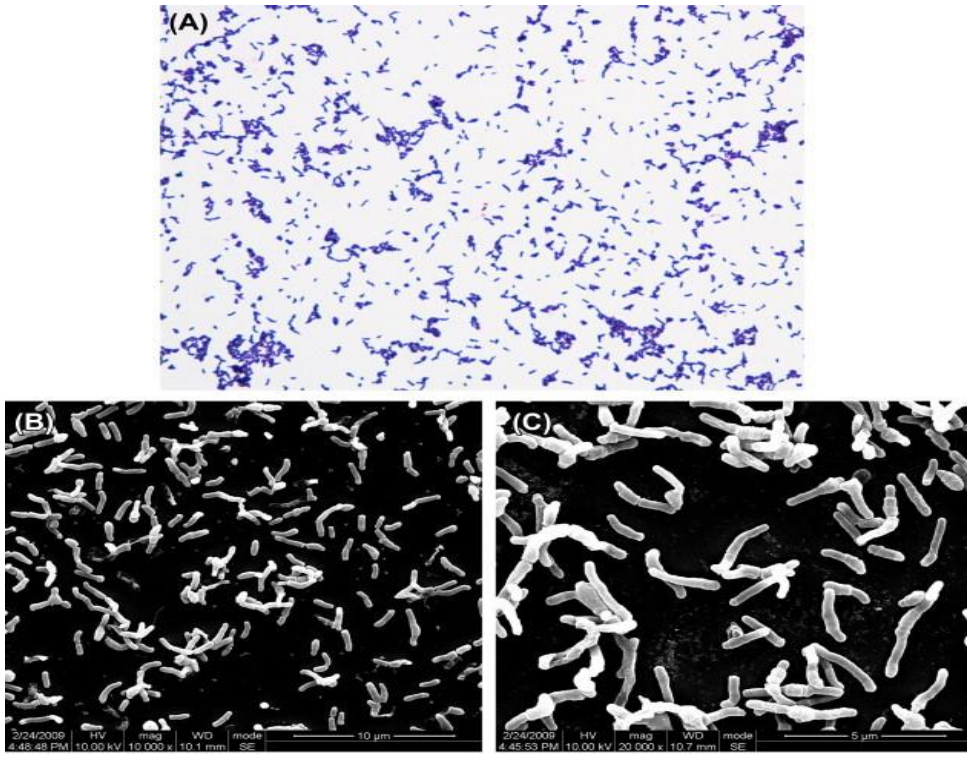


Figure 1.3: (A) *Propionibacterium acnes* cells (Gram stain). (B) *P. acnes* cells (SEM). (C) *P. acnes* cells (SEM). *P. acnes* cells are polymorphic but are mainly thin and long rod-shaped cells. H.R. Jalian, ... J. Kim, in Comprehensive Medicinal Chemistry II, 2007 from molecular medical microbiology second addition 2015 ,science direct .

Acne is a chronic and multifactorial inflammation of the skin. It is associated with a decreased number of *S. epidermis* and an over colonization by selected *P. acnes* phylotypes in the sebaceous unit, leading to different levels of activation of the innate immunity, thus resulting in different severity levels of inflammatory acne. Recent research seems to confirm the beneficial role of *S. epidermis* in the physiopathology of acne through limiting *P. acnes*-induced colonization of the skin and of inflammation (Claudel et al, 2019).

1.4.3 Epidemiology

Acne Vulgaris is a major skin disease affecting young adults worldwide. It affects around 80% of teenagers with similar incidence in both sexes within this age group. The majority of epidemiological studies on Acne Vulgaris focus on the age of

adolescence. Based on published reports, it appears to range from 44.1% in 1,857 adolescents in Peru and 49.8% in 317 adolescents in the UK to 67.3% in 9,570 adolescents in New Zealand, 82.1% in 1,290 adolescents in Portugal, 83.1% in 666 adolescents in Australia, 87.9% in 1,045 adolescents in Singapore, 91.3% in 522 adolescents in China (Hong Kong), and 94.9% in 594 adolescents in Belgium (Zouboulis et al., 2014).

Acne Vulgaris also occurs in adulthood where it affects the patient's quality of life and, in many cases, employment chances. Unemployment is higher in individuals having Acne compared to those who do not. Studies discussing the epidemiology and psychosocial effects of Acne occurring in adulthood covered university students and other young adults, whereas other studies were more concerned with female adults with Acne. The prevalence of Acne in adulthood has been reported to be 56.2% in a study of Saudi medical students. The prevalence of Acne was 50.9% for men and 42.5% for young female adults in their twenties who were not university students (Zouboulis et al., 2014).

Gender differences in the presentation of Acne seem to be more pronounced in adulthood. Such differences are noted in the disease's prevalence, effect on the quality of life, treatments choices, and clinical presentation. A cross-sectional and longitudinal questionnaire study of 60 adults suffering from Acne indicated that the patients experienced undesirable effects on their functionality and emotional well-

being related to their skin condition that were comparable to those experienced by patients suffering from psoriasis (Zouboulis et al., 2014).

1.4.4 Virulence Factors of *P. acnes*

With the sequencing of the first *P. acnes* isolate in 2004 (KPA171202, a type IB strain recovered from skin), a number of putative virulence genes were identified with assigned roles in tissue degradation, cell adhesion, inflammation, and polysaccharide biosynthesis for biofilm formation. Ever since then, various genetic components particular to each lineage have been identified; this might aid in the explanation of the differences in function between lineages and how this relates to different disease states. The presence of the clustered regularly interspaced palindromic repeats (CRISPR)/Cas locus in health-associated type II strains is considered an interesting genetic variation among *P. acnes* lineages. This arrangement is only partly present in type III and possibly has no function. However, it is entirely nonexistent in type I strains. The presence of CRISPR in *P. acnes* could avert the bacteria from acquiring extra genetic elements which might foster virulence and Acne pathogenesis as CRISPR is known as a bacterial adaptive immune system against viruses, phages, and foreign DNA. For instance, a linear plasmid found in acne-associated type I strains was discovered which carries a tight adhesion (tad) locus, this feature has been seen in many pathogens and is necessary for biofilm

formation, colonization, and virulence. Similarly, a unique genomic island (locus 4), is present in the acne-associated strains of RT8, representing type IB, that encodes a sequence of enzymes which produce a class of biologically active natural compounds called non-ribosomal peptides (NRP). Other NRP metabolites were reported to have a role in cell-mediated toxicity and iron sequestration in addition to potential activity against bacteria and fungi. The discovery of genetic mutations of possible importance in many genes encoding triacylglycerol lipases, essential for sebum degradation, is another reasonable justification for the selectivity of type II strains to healthy skin.

The analysis of *P. acnes* strains obtained from patients suffering from Acne Vulgaris showed various large genomic islands encoding for genes corresponding with Streptolysin S biosynthetic cluster that plays a role in the biosynthesis and transport of bacterial toxins in addition to other genes that are assumed to have a role in virulence, cell survival, and transport. On the other hand, analysis of the genetic material of P. acnes strains found on healthy skin had an abundancy of genes related to carbohydrate and lipid metabolism and the biosynthesis of nutrients rather than genes related to virulence (O'Neill & Gallo, 2018).

1.4.5 Pathophysiology of Acne Vulgaris

The microbial community occupying the surface of the skin mainly constitutes bacteria that belong to the genera of *Staphylococci*, *Propionibacteria*, and

Corynebacteria. Maintaining a healthy skin state requires a healthy interaction between members of the cutaneous microbiota. Whereas *P. acnes* is considered a commensal bacterium, mostly found in sebaceous units, and plays a crucial role in regulating skin homeostasis and averting the colonization of harmful pathogens, it displays an opportunistic behavior in the pathogenesis of Acne Vulgaris (Dréno et al., 2018). Since *P. acnes* bacteria are normally found on the skin of a great number of people regardless of the presence of acne lesions, defining chronic inflammatory Acne as an infectious disease does not seem plausible. The presence of *P. acnes* seems to only help set off the skin disease on condition that certain dermato-physiological parameters are met. Colonization by *P. acnes* is crucial for the foundation of the pathology, but is not enough. Four factors are considered to be the major key players in the pathophysiology of Acne Vulgaris; (1) androgen-induced seborrhea, (2) hyperkeratinization, (3) obstruction of the follicular epithelium, (4) and the proliferation of P. acnes, which eventually lead to inflammation. The process by which a pilosebaceous follicle transforms into a primary acne lesion, the comedone, is called comedogenesis. The process starts with the increased secretion of sebum, known as seborrhea, and the hyper keratinization that follows. *P. acnes* frequently becomes enclosed within layers of coneocytes and sebum during comedogenesis and very quickly colonizes the comedone leading to the formation of a microcomedone, which is invisible to the naked eye. Microcomedones can grow

larger and become a comedone, which has two distinct presentations; (1) a white head, in which the comedone is unable to escape the top layer of cell debris, sebum, and *P. acnes* and its products, thus remains closed and appears on the surface of the skin as a coloured bump that is prone to inflammation and rupture, (2) a black head, in which the comedone is open to the skin surface. When comedones rupture and their follicular content spreads into the dermis and not the skin surface, inflammatory acne forms. Inflammatory acne lesions are categorized into papules, pustules, and nodules based on the extent of damage exerted on the wall of the comedone. The most severe of all acne lesions is the nodule, which may end up in scarring as with any other form of severe inflammatory acne (Dréno et al., 2018).

1.4.6 Emergence of Antibiotic Resistance in *P. acnes*

Resistant strains of *P. acnes* were described in many studies. It has been reported that the clinical manifestations and numbers of *P. acnes* were linked to the emergence of resistant strains that were identified as having a ten times increase in minimum inhibitory concentration (MIC) to tetracycline and erythromycin (Leyden et al., 1983).

1.5 Antimicrobial Susceptibility Testing

The objective of antimicrobial susceptibility testing is to determine the extent of sensitivity and resistance of microorganisms against antimicrobial agents.

Furthermore, the antimicrobial susceptibility profile of an organism is a key element for identifying the isolated microorganism (Forbes et al., 2007). The impact of environmental factors should be controlled, in order to perform the antimicrobial susceptibility test correctly to obtain accurate results the conditions for testing should be strictly and extensively standardized. The terms susceptible (S), intermediate (I) and resistant (R) are used to indicate if the tested antimicrobial agent is likely to be effective therapeutically against a specific organism, and defined according to The Clinical & Laboratory Standards Institute (**CLSI**) as follows (CLSI,2005 ; HPA, 2008):

- Susceptible: Which implies that an infection due to the strain may be appropriately treated with antimicrobial agent at a dosage recommended for that type of infection and infecting species, unless otherwise contraindicated.
- Intermediate: This category includes isolates with antimicrobial minimum inhibitory concentrations (MIC) that approach usually achievable blood and tissue concentrations and for which response to chemotherapy may be variable. Microorganisms with intermediate susceptibility may be eradicated from body sites where the drugs are concentrated or when an increased dosage of a drug can be used. In practice the intermediate category is often not reported, except with a few antibiotic combinations, and the intermediate and resistant categories are combined as resistant to avoid uncertainty.

- Resistant: Strains in this category are not inhibited by the usually achievable concentrations of the agent at the site of infection and/or have specific resistance mechanisms. Therapy is highly likely to fail.

The choice of the right susceptibility testing method is based on the type of microorganism, technical simplicity, required accuracy, applicability to working practices in the individual laboratory and cost.

Variety of susceptibility testing methods includes: Disc diffusion method, Dilution methods, Breakpoint methods, Etest, Automated methods, Detection of mechanism of resistance and Molecular methods.

1.6 Protein Purification

Protein purification ranges from simple one-step precipitation procedures to large scale validated production processes. Oftentimes, more than one purification step is needed to achieve the desired level of product purity, but every step of the process will result in some loss of the product. To get successful and efficient protein purification it is necessary to select the most appropriate techniques, optimize their performance to suit the requirements, and combine them in a logical way to increase the yield and limit the number of steps required (GE healthcare, 2001). The "Three-Phase Purification Strategy" is widely used in the development of purification processes for therapeutic proteins in the pharmaceutical industry. This strategy guarantees faster method development, shorter time to pure product, and is

economically efficient. The complete purification process should consist of: (1) Sample Preparation: primary isolation of the target protein from the source material, (2) Capture Phase: Isolate, concentrate and stabilize the target product, (3) Intermediate Purification: Remove most of the bulk impurities, (4) Polishing: Achieve high purity by removing any remaining trace impurities. However, it is not obligatory for all purification strategies to include all four purification steps. Purity demands for the product or bulk impurities may be very low, that the capture and intermediate purification may be achievable in a single step. Most purification protocols include some form of chromatography as it has become an effective tool for protein purification. Proteins are purified by various chromatographic purification techniques that separate materials according to differences in specific properties (GE healthcare, 2001).

In this research project antibodies fraction were purified by Protein G (PG) and Protein A (PA) Chromatography while PGRPs and Lactoferrin were isolated and characterized using Heparin Affinity Chromatography.

1.7 Research Background

Acne vulgaris, caused by the bacterium *P. acnes,* is the most common cutaneous disorder in adolescents. The disease affects mostly the sebaceous glands leading to characteristic inflammatory lesions called comedones in the back, face and trunk

(Abu-qatouseh et al., 2019). Acne is not a life-threatening disease, however, it has significant psychological and physical effects such as poor self-image, depression, anxiety, social inhibition and permanent scarring. *P. acnes* is capable of interacting with the components of the immune system and in biometabolize sebum fats into free fatty acids, leading to inflammatory response through chemotaxis of neutrophils and monocytes inductions to produce various pro-inflammatory mediators, such as; IL-8 and TNF (Kim, 2005). Topical therapy is advisable in acne treatment in mild to moderate acne conditions. Recently, available treatments for acne are mostly based on antibiotics and retinoids. The use of antibiotics has a lot of limitations due to development of resistance by bacteria. On the other hand, retinoids are highly teratogenic, therefore topical acne treatment is of high interest. Due to both direct and indirect effects on the pathogenic aspects and the severity of the acne lesions, therapeutic success in acne is not always guaranteed (Dessinioti & Katsambas, 2017). Major drawbacks associated with the usually used topical agents for the treatment of acne include, but are not limited to the patient compliance since the treatment usually lasts for long periods. Antibiotics and isotretinoin constitute first line of treatment options for acne. However, bacterial resistance and teratogenicity of isotretinoin led scientists to search for safer therapeutic options and more potent (Walsh et al., 2016).

Animal milk has been used in the preparation of pharmaceutical and cosmetic compositions such as anti-acne products. However, a drawback to its use is the wide-spread allergy to individuals in some populations. It was found that camel milk composition is closer to the human milk. Passive immunity is provided to newborns by Immunoglobulins present in colostrum until its own immune system matures. The concentration in colostrum of specific antibodies against pathogens can be raised by immunizing a mammal with these pathogens or their antigens. Immunized milk products are preparations made of such hyper-immune antibodies enriched from it (Al-Qaoud et al., 2014).

Camel milk and milk derived products have a growing potential in pharmacy, food and biotechnology industry and its therapeutic value has been investigated and documented in many studies before (Al-Qaoud et al., 2014).

The findings of this research imply that the constituents of immunized camel milk whey (i.e. polyclonal anti-*P. acnes* antibodies, peptidoglycan recognition proteins (PGRPs), and lactoferrin) possess significant antimicrobial and anti-inflammatory activity against *P. acnes*. Topical formulae including; facial wash, serum, cream and gel enriched with immunized camel milk whey were developed and their activity against *P. acnes* was evaluated. In conclusion, immunized camel milk whey that has been developed in this research exerts significant antimicrobial and anti-

inflammatory activity against *P. acnes* and the unique composition formulated has been patented.

The workflow adopted in this research project is summarized in the following scheme:

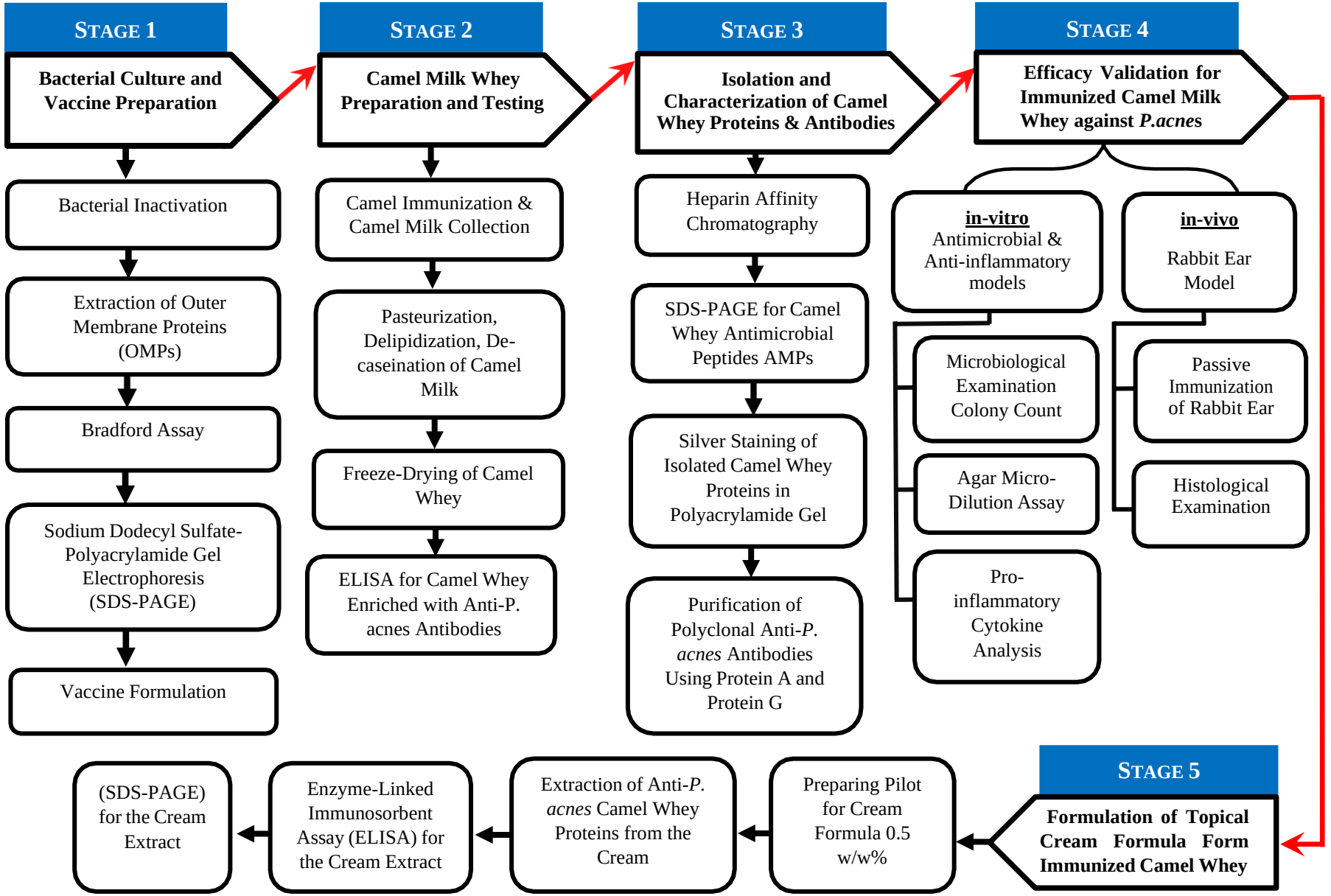


Figure 1.4 : Workflow of this research project.

1.8 Research Objectives

The objectives of this research work were; firstly, to evaluate the antimicrobial effects of immunized camel milk whey and its components against *P. acnes.* Secondly, to evaluate the anti-inflammatory effects of immunized camel milk whey and its components against *P. acnes*. Thirdly, to compare the anti-microbial and anti-inflammatory properties of immunized and non-immunized camel milk whey against *P. acnes*. Finally, to develop a pharmaceutical formula that harnesses all the unique beneficial properties of Camel milk whey against Acne Vulgaris.

The project started by heat-killing cultured *P. acne*s in order to obtain their outer membrane proteins (OMPs) acting as antigens. The OMPs were mapped using SDS-PAGE to confirm their presence, and quantified using Bradford Protein Assay. Thereafter, OMPs were used to prepare a vaccine that is later on injected into female lactating camels in timely manner. Immunized camel milk was collected then pasteurized, delipidized, de-caseinated, and freeze-dried to obtain the whey into powder form. The anti-*P.acnes* antibodies in the immunized camel whey were mapped using SDS-PAGE to confirm their presence, and quantified using ELISA. Then, the antimicrobial peptides (AMPs) including PGRPs and Lactoferrin were isolated and characterized using Heparin Affinity Chromatography, and mapped using SDS-PAGE. The anti-microbial and anti-inflammatory activity of the

immunized camel whey and its constituents against *P. acnes* was evaluated in vitro. In vivo activity was also evaluated using rabbit ear model. After the activity of the immunized camel whey was confirmed, four topical formulae were developed and their composition and preparation protocol were patented.

Chapter 2: Materials and Methods

2.1 Materials

The following chemicals and materials were used in this research:

Potassium Phosphate (KH_2PO_4) from Acros Organics, USA. Sodium Azide (NaN_3) from BDH Chemical Ltd, Poole, UK. Titramethethylenediamine (TEMED) from BIO Basic Inc, Canada. Phosphate Buffered Saline 1X (PBS) from EuroClone, Italy. Potassium Chloride from Fisher Scientific, New Hampshire, USA. Brilliant Blue, Coomassie® Brilliant Blue G 250, and N, N´-Methylenebisacrylamide from Fluka Chemie AG, Switzerland. Mueller-Hinton Agar from Oxoid Ltd., England. Bovine Serum Albumin (BSA) from PAA Laboratories GmbH, USA. Formaldehyde 37% Silver Sequence™, Formaldehyde 37% Silver Sequence™, Silver Nitrate ($AgNO_3$), Sodium Carbonate (Na_2CO_3), and Sodium Thiosulfate Silver Sequence ™ (10 mg/mL) from Promega Corporation, USA. Ethanol 95%, Orthophosphoric Acid, Sodium chloride (NaCl), Sodium dihydrogen phosphate-2-hydrate (NaH_2PO_4 * 2 H2O), Sodium phosphate dibasic dehydrate (Na_2HPO_4 * 2 H_2O) from Riedel-de Häen, Bucharest, Romania. 0.1% O-phenylenediamine, Acrylamide, Ammonium per Sulfate (APS), Bromophenol Blue, Glycine, Sigma Marker™ Wide Range, Sodium Dodecyl Sulfate (SDS), Trizma base, Trizma hydrochloride, and β-Mercaptoethanol from Sigma - Aldrich Chemie GmbH, Taufkirchen, Germany. Methanol 90% and Glycerol 10% from Tedia Company Inc, Ohio, USA. Toyopearl® AF-Heparin HC-650 column (1 ml) from Tosoh Bioscience LLC,

Japan. Microbial Rennet Valiren® from Valiren, North Carolina, USA. Acetic Acid 100% from VWR International LLC, Missouri, USA.

2.2 Methods

The methodologies utilized in this research could be divided into five main stages based on workflow successiveness, previously illustrated in Figure 1.4, as detailed below:

2.2.1 Stage I: Bacterial Culture and Vaccine Preparation

2.2.2 Stage II: Camel Milk Whey Preparation and Testing

2.2.3 Stage III: Isolation and Characterization of Camel Whey Proteins and Antibodies

2.2.4 Stage IV: Validation (*In vitro* and *In vivo*)

2.2.5 Stage V: Formulation of a Topical Cream Formula from Immunized Camel Whey

2.2.1 Stage I: Bacterial Culture and Vaccine Preparation

2.2.1.1 Bacterial strains and Growth conditions

A standard strain of *P. acnes* (NCTC 737), donated by the Jordan Company for Antibody Production (MonoJo), were cultured on Mueller Hinton Agar (MHA) (BBL, Japan) under anaerobic conditions using Gas-Pak (Oxoid, UK) at 37ºC for 72 hours.

2.2.1.2 Bacterial Inactivation

P. acnes bacterial cells were harvested by adding 1 mL of 1x autoclaved Phosphate Buffered Saline (PBS) to the growing bacteria, and collected from the plate with a sterile Pasteur pipette. The bacteria were washed three times with PBS, and the suspension was prepared as to 2 McFarland standard/1.5 mL. Bacterial cultures were inactivated by heat killing at 80°C for 30 minutes and centrifuged at 5000 xg for 5 minutes. Bacteria were mixed with PBS until reaching the required 2 McFarland/1.5 ml.

Finally, 1 ml of the culture was streaked over a Muller Hinton Agar (BBL, Japan) plate. The plates were incubated at 37°C for two weeks, and were checked on regular basis and lack of growth on the plates were used as an indicator of complete inactivation of the bacteria.

2.2.1.3 Extraction of Outer Membrane Proteins (OMPs)

P. acnes plates were scrapped using sterile Phosphate Buffer Saline (PBS), and the pellet was washed twice with PBS. Then, the pellet was suspended into 15 ml of autoclaved PBS, and was sonicated as follows; 15 minutes "Boost", 15 minutes "Sweep", 15 minutes "Degas", in ice cold water, and this last step was repeated 15 times. The suspension was centrifuged at 5000 rpm for 15 minutes, and the resultant supernatant was filtered using 0.2 micrometer syringe filter, and the resultant filtered supernatant was concentrated using Vivaspin 30 kDa cut-off. Finally the 15 ml of the *P. acnes* OMPs was obtained. Protein concentration was determined using Bradford Protein Assay (as described in section 2.2.1.4 of this chapter), and SDS-PAGE was used to map the protein profile of the OMPs (as described in section 2.2.1.5 of this chapter).

2.2.1.4 Measurement of Protein Concentration using Bradford Protein Assay

2 mg/ml Protein Standard (Sigma code no. P0834) was used as a reference protein. Coomassie Stock Reagent (5x Solution) was prepared as follows; 100 mg of Coomassie Brilliant Blue G250 were dissolved in 50 mL of 95% ethanol, and then 100 mL of ortho-phosphoric acid were added. The solution was stored at 2-8^{o}C in the refrigerator. For Coomassie working solution, the stock reagent was diluted five times with distilled water, and then filtered through (Whatman no. 1) filter paper.

For Standard Curve protein concentration, 2-fold serial dilution from the protein standard (2 mg/ml BSA) was used. In 96 well plates, 50 µl PBS solution were dispensed to all the wells across the row of the plate. Then, 50 µl of the standard solution were added and mixed to the first well, this is the first 2-fold dilution. Afterwards, the micropipette with the same tip was used to carry out a second 2-fold dilution. This was carried consecutively until the third last well of the plate. The last four wells were used as blank (only PBS). For the *P. acnes* protein samples, 20 µL of each sample were prepared at different concentrations using 2-fold serial dilution. Then, 200 µl of Coomassie working solution were added to each well (standard, tested *P. acnes* samples, and blanks). The plate was mixed on the orbital shaker for 10 minutes and then the absorbance at 595 nm were recorded with a MultiScan GO Spectrophotometer.

2.2.1.5 Mapping of the protein Profile of the OMPs Using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

OMPs were separated by the standard SDS-PAGE method. In brief, 0.5 mm thick 10% acrylamidebisacrylamide gels under non-reducing conditions were used. Resolving gel composed of 4 mL distilled water, 2.5 mL resolving buffer, and 10 µL TEMED were firstly prepared. Stacking gels (4%) were prepared by adding 6 mL of distilled water, 2.5 mL stacking buffer (pH 6.6), 1.3 mL of 30% acrylamide-bisacrylamide solution, 100 µL of 10% Ammonium persulfate (APS), and 10 µL

TEMED. Protein fractions were mixed with an equal volume of sample buffer lacking β-mercaptoethanol (pH 6.8). Molecular weight protein standard ladder was used for estimation of band sizes. Electrophoresis conditions included running buffer with pH 8.3, 120 volts and time of 60-120 min. The gels were visualized after staining with 0.2 % Coomassie brilliant blue R-250 and de-stained with 20% acetic acid until clear bands were seen.

2.2.1.6 Vaccine Formulation

The vaccine was formulated by mixing 1.5 mL of *P. acnes* antigens (OMPs 500µg/Dose) with 1.5 mL of Alum adjuvant resulting in a total of 3 ml. The vaccine was gently mixed for at least 10 minutes using the double hub needle. The vaccine was ready for use when a milky emulsion was formed.

2.2.2 Stage II: Camel Milk Whey Preparation and Testing

2.2.2.1 Camel Immunization Protocol and Camel Milk Collection

The vaccination was given subcutaneously at two different sites to lactating female camels in timely manner. Milk and blood samples were collected from the camels before and after the vaccination. Blood samples were collected on day 0, 9, 18, 34, 48 and 74 from each camel using sterile syringes. All samples were centrifuged at 3300 x g for 10 minutes at 4°C. Vaccine boost shots were given to further provoke the immune system of camels and raise the antibody titer.

2.2.2.2 Pasteurization of Camel Milk

The camel milk was pasteurized at a temperature of 65±1 ◦C for 30 minutes in the laboratory using a water bath (JEIO TECH, Seoul, Korea). Pasteurization timing should be started when the milk temperature measurement is at 65 ◦C. After time elapsed, the milk should be left at room temperature to cool down.

2.2.2.3 De-lipidization of Camel Milk (Skimming)

To delipidize (skim) the milk, the milk fat should be separated by centrifugation at 10000 x g for one hour at 0 ºC. Two hundred milliliters of pasteurized and non-pasteurized whole camel milk were centrifuged at 10000 × g for 1 h at 0 ºC. The top layer (cream) should be then separated manually.

2.2.2.4 De-caseination of Camel Milk

After skimming, casein should be removed by precipitation. Firstly, milk should be warmed up to reach 37 ºC, then microbial cheese rennet Valiren® (100 mg/L) should be gradually added to the milk while stirring. Afterwards, casein will be left to precipitate for 30-60 minutes. Finally, casein should be filtered using a gauze. A second centrifugation for 10 minutes was done to further remove the remaining casein.

2.2.2.5 Freeze-Drying of Camel Whey

Freeze drying is a water removal process typically used to preserve perishable materials, to extend shelf life or make the material more convenient for transport. Freeze drying works by freezing the material, then reducing the pressure and adding heat to the frozen water in the material to sublimate (Duroudier, J. P., 2016). Freeze-drying was performed using a laboratory freeze dryer VaCo 2 (Zirbus, Bad Grund/Harz, Germany). 50 ml of whey were poured in a glass pulp. Pulps were then immersed in liquid nitrogen with whirling till the whey completely froze at a fixed temperature of -50 ◦C for 24 hours.

2.2.2.6 Enzyme-Linked Immunosorbent Assay (ELISA) for Camel Whey Enriched with Anti-*P. acnes* Antibodies

Indirect Enzyme-Linked Immunosorbent Assay (ELISA) was used to measure polyclonal antibodies in Camel milk against *P. acnes*. Briefly, 96-well microplates (Greiner, Germany) were coated with 100 µl of antigen (10 µg/ml *P. acnes* antigens) in carbonate-bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. Afterwards, plates were washed 3 times with 100 µl of 0.15 M PBS (pH 7.2) containing 0.05% Tween 20 and then were blocked with 200 µl of 2% bovine serum albumin (BSA) in PBS. An amount of 100 µl of the immunoglobulin fraction isolated from whey protein preparations samples diluted at 1:100 in 1% BSA/PBS

were added in duplicates and incubated for one hour at room temperature. Each run included positive and negative samples as control. Enzymatic detection system made of horse radish peroxidase (HRP) conjugated protein A and protein G diluted at 1:1000 in 1% BSA/PBS and 0.1% O-phenylenediamine containing hydrogen peroxide in 0.1 M citrate buffer (pH 4.5). The absorbance was measured at 490 nm using ELISA reader (AsysHitech, Switzerland).

2.2.3 Stage III: Isolation and Characterization of Camel Whey Proteins and Antibodies

2.2.3.1 Heparin Affinity Chromatography

Camel whey protein fractions were purified using a 1 ml Toyopearl® AF-Heparin HC-650 column on Bio-Rad (BioLogic LP, California, USA) purification system. Sodium Phosphate Buffer (0.01 M) and Sodium Chloride (0.1 M) were used as binding buffer at pH 7.4. The samples were diluted at (1:1) with the buffer and subsequently filtered through a 0.45 μm filter to adjust them to the composition of the binding buffer before loading. Sodium Chloride (0.7 M) was used as an elution buffer at pH 7.4. Elution was carried out in a linear gradient from 0.17-0.35 M Sodium Chloride at ambient temperature.

The purification process was proceeded as follows; (1) buffers were degassed and filtered through a 0.45 μm syringe filter, (2) all tubes were washed with distilled water and positioned correctly into the fraction collector (Bio-Rad, Model 2110 Fraction Collector, California, USA), (3) purification system tubes were washed with distilled water to remove any trapped traces or bubbles, (4) the purification system was set on “Purge” at maximum flow rate and washed with binding buffer A (consisting of; Sodium Phosphate Dibasic Dehydrate 0.005 M, Sodium Dihydrogen Phosphate-2-Hydrate 0.005 M, Sodium Chloride 0.1 M, and distilled water sufficient for a final volume of 1000 ml) to wash away the distilled water, (5) the 1 ml

Toyopearl® AF-Heparin HC-650 column was fixed onto the system and the UV lamp was turned on and set on zero, (6) binding buffer A was run at 1 ml/min flow rate until the conductivity was stabilized, (7) Biologic LP software was then turned on and 5 ml of the sample containing filtered and skimmed camel milk diluted with the same volume of binding buffer A and filtered through 0.45 μm syringe filter, were loaded, (8) the washing buffer was collected to be analysed later by SDS-PAGE, and the column was washed for 10 minutes until the UV reading was zero, (9) binding buffer A and elution buffer B (consisting of; Sodium Phosphate Dibasic Dehydrate 0.005 M, Sodium Dihydrogen Phosphate-2-Hydrate 0.005M, Sodium Chloride 0.7 M, and distilled water sufficient for a final volume of 1000 mL) was run in a linear gradient from 0.17-0.35 M Sodium Chloride, (10) 80 fractions were collected at 0.5 ml each resulting in a total volume of 40 ml, and the column effluent was monitored at 280 nm, (11) following the last fraction collection, regeneration buffer (consisting of; 2 M Sodium Chloride and distilled water sufficient for a final volume of 500 mL) was run on the column, (12) storage buffer (consisting of; Sodium Chloride 0.5 M, Sodium Azide 0.02% and distilled water sufficient for a final volume of 500 ml) was run for storage purposes, (13) finally, the column was removed and stored at 4 ^{o}C, the system tubes were washed with distilled water at "Purge", and the pump tubes were loosed. The collected fractions were stored at 4 ^{o}C.

2.2.3.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) for Camel Whey Antimicrobial Peptides (AMPs)

Components of camel whey protein isolates were separated by the standard SDS-PAGE method. Briefly, 0.5 mm thick 10% acrylamidebisacrylamide gels under non-reducing conditions were used. Resolving gels of 4 ml distilled water, 2.5 mlL running buffer Tetramethylethylenediamine (TEMED) were firstly prepared. Stacking gels (4%) were prepared by adding 6 ml of distilled water, 2.5 ml stacking buffer (pH 6.6), 1.3 ml of 30% acrylamide-bisacrylamide solution, 100 µl of 10% Ammonium persulfate (APS), and 10 µl TEMED. Protein fractions were mixed with an equal volume of sample buffer lacking β-mercaptoethanol (pH 6.8). Molecular weight protein standard ladder was used for estimation of band sizes. Electrophoresis conditions included running buffer with pH 8.3, 120 volts and time of 60-120 min. The gels were visualized after staining with 0.2 % Coomassie brilliant blue R-250 and washing with 20% acetic acid until clear bands were seen.

2.2.3.3 Silver Staining of Isolated Camel Whey Proteins in Polyacrylamide Gel

The Polyacrylamide gel is placed in a shallow plastic tray, and 200 ml of fix/stop solution (consisting of; 5 ml Acetic Acid 100% and 450 mL of Ultrapure Water) are poured, then, the gel was agitated well for 60 minutes. Afterwards, the gel was rinsed with Ultrapure Water three times (2 minutes each) with agitation, and the gel was

allowed to drain for 10-20 seconds between rinses. A staining solution (consisting of; 0.2 g Silver Nitrate, 300 µl Formaldehyde 37%, and 200 ml of Ultrapure Water) was poured and the gel was agitated for 30 minutes. The staining solution was then discarded and the gel was quickly rinsed with Ultrapure Water, drained, and 200 ml of chilled developing solution (consisting of; 12 g Sodium Carbonate, 600 µl, 80 µl Sodium Thiosulfate (10 mg/ml), and 400 ml of Ultrapure Water) were poured around the gel, this step was carried out in no longer 5-10 seconds. The gel was subsequently well agitated until the bands started developing and the solution turned yellow. The developing solution was then discarded and the remaining 200 ml of chilled developing solution were poured on the gel and developing was continued for 1-2 more minutes until all bands became visible. Finally, the developing solution reaction was terminated and the gel was fixed by adding 200 ml of fix/stop solution directly to the developing solution. The gel was rinsed twice with Ultrapure Water and stored in the remaining 100 ml of fix/stop solution.

2.2.3.4 Purification of Polyclonal Anti-*P. acnes* Antibodies Using Protein A and Protein G Chromatography

Protein G (PG) and Protein A (PA) Chromatography were used to isolate polyclonal immunoglobulin subclasses (IgG1, IgG2, and IgG3) from immunized Camel milk whey. Briefly, 500 mg of immunized Camel whey powder were dissolved in 50 mL of binding buffer consisting of; 20 mM Sodium Phosphate Buffer at a pH value of

7.15 (10mg/mL powder ≈ 2 mg/mL total protein), and were subsequently injected into a 5 mL Hitrap™ Protein G-Sepharose column (GE HealthCare, Wisconsin, USA), the flow was subsequently applied to a 5 mL Hitrap™ Protein A-Sepharose column (GE HealthCare, Wisconsin, USA). The bound proteins were eluted with elution buffer consisting of 0.1 M glycine buffer at a pH value of 2.7. Neutralizing buffer consisting of; 1 M Tris-HCl at a pH value of 9.0, was used to neutralize the pH of each collected fraction directly after the collection. All peaks were finally concentrated using Vivaspin® 10 kDa membrane cut-off column (Sartorius, Germany). All buffers used were filtered using 0.45 µm syringe filter, and degassed using the SoniClean sonicater for 15 minutes using the degassing program. The flow rate was kept at 2 mL/minute, and 2 mL fraction volumes were collected. SDS-PAGE of the purified immunoglobulins was carried out as described previously in section 2.2.3.2 of this chapter.

2.2.4 Stage IV: Validation (In vitro and In vivo)

2.2.4.1 In Vitro Testing of the Antimicrobial and Anti-inflammatory Efficacy of Immunized Camel Whey against *P. acnes*

2.2.4.1.1 Microbiological Examination

A standard inoculum of *P. acnes* was mixed with immunized Camel whey and subsequently incubated for 72 hours. A second standard inoculum of *P. acnes* was not mixed with immunized Camel whey and was labeled as negative control. After

incubation, the total count of colony forming units (CFUs) was determined and compared with the negative control.

2.2.4.1.2 Agar Micro-Dilution Assay

Serial dilutions of the isolated; Peptidoglycan Recognition Proteins (PGRPs), lactoferrin, and anti-acnes polyclonal antibodies, were prepared at concentrations starting from 0.05 to 1 mg/mL, and were incubated for 5 hours with *P. acnes* bacteria in Sodium Phosphate Buffer (pH 7.2), consisting of 1% trypticase soy broth and glucose. The rest of the bacteria were plated on Tryptic Soy Agar (TSA) with glucose (Oxoid, UK) agar plates, and colony-forming units (CFUs) were counted after incubation for 3 days at 37 ^{o}C.

2.2.4.1.3 Pro-inflammatory Cytokines Analysis

An amount of 0.1 ml of heat-killed *P. acnes* (as inflammation control), and 0.1 ml of a solution consisting of heat-killed *P. acnes* mixed with different concentrations of isolated camel whey protein fractions (0.01 to 1 mg/ml), were used to stimulate seeded human monocytic THP-1 cells (Thermo Fischer Scientific, UK) prepared at $1x10^6$ cells/ml in 24 well plates with serum-free medium. The plates were incubated for 24 hours. After incubation time elapsed, cell-free supernatants were collected,

and TNF-α, IL-1α, and IL-8 were analyzed by Enzyme-Linked Immunosorbent Assay (ELISA) as described in section 2.2.2.6 of this chapter.

2.2.4.2 In Vivo Efficacy Testing for the Whey Protein Concentrate of the Milk of Immunized Camels against *P. acne*s Using Rabbit Ear Model

P. acnes (10^8 CFU/ml (OD_{600} = 2)) bacteria were mixed with the whey protein concentrate and incubated for 2 hours before intradermal injection in the central portion of rabbit ear. Both positive whey and negative whey were used and compared. The positive control was bacteria with PBS, while negative control was the whey of non-immunized camel. Injection was gradually performed by 28 gauge needle to prevent leakage. Gross examination was performed daily along the period of investigation (two weeks). The testing included Gross Examination (Ear inflammation, redness, heat, thickness, and papule and pus formation) and Histopathological evaluation was performed after sacrificing the animals (Inflammatory cells types and density, micro abscess formation, ulceration).

2.2.4.2.1 Bacterial Culture Preparation

*P. ac*nes were cultured on Mueller Hinton Agar (MHA) under anaerobic conditions using Gas-Pak (Oxoid) at 37 ^oC for 72 hrs. Standard inoculums of $1x10^{10}$ CFU/ml are prepared by inoculating colonies of *P. acnes* in PBS.

2.2.4.2.2 Induction of *P. acnes* Inflammation

An amount of at least $1x10^{10}$ CFU/ml of *P. acne*s (30, 50, 100 µl) in PBS was intradermally injected in the central portion of the right ear. As a control, purified PBS was injected into the left ear of the same rabbit. To prevent leakage, bacteria were gradually injected into rabbit ears using a 28-gauge needle followed by a slow withdrawal of the needle.

2.2.4.2.3 Histological Examination

For histological observation, the ear was cross-sectioned, stained with hematoxylin and eosin (H&E) and viewed on a Zeiss microscope.

2.2.4.2.4 Passive Immunization of anti-*P. acnes* Whey Protein from Camel Milk against *P. acnes* Induced Inflammation

P. acnes will be pre-treated with 5% (v/v) anti acne whey protein in the medium at 37 ^{o}C for 2 hours. The rabbit ears will be injected intradermally with amounts of 50, 100 µl aliquots of anti- acne neutralized *P. acnes* (1×10^{10} CFU/ml) suspended in PBS. As a control, similar quantities of PBS were injected into the left ear of the same rabbit. The increase in ear thickness will be measured using a micro-caliper (Mitutoyo, Japan) after the bacterial injection, the increase in ear thickness of *P. acnes* injected ear is calculated as % of a PBS-injected control.

2.2.4.2.5 Histological Examination Following Passive Immunization

For histological observation purposes, 11 days after the injection, the ear is excised, cross-sectioned, stained with H&E, and viewed on a microscope.

2.2.5 Stage V: Formulation of a Topical Cream Formula from Immunized Camel Whey

2.2.5.1 Preparing a Pilot for the Cream Formula 0.5 w/w%

The cream formula was prepared, as demonstrated in the patent (2014/0072648), as follows; (1) Cetyl Alcohol, Glyceryl Monostearate and MERKUR 791 were molten in the same container at 75 ^{o}C using a water bath, (2) Tween-20 was mixed with purified water, (3) Camel whey powder was dissolved in the Tween-20 mixture, (4) the mixture in step 3 was heated up to 45-50 ^{o}C, (5) the mixture in step 1 was cooled down to 65-70 ^{o}C, (6) finally, both mixtures were mixed together and cooled down while mixing until the cream turned white.

2.2.5.2 Extraction of Anti-*P. acnes* Camel Whey Proteins from the Cream

One gram of the cream enriched with antibodies derived from camel milk was mixed with 1mL of Phosphate Buffered Saline (PBS) in a 2 ml Eppendorf tube using a small spatula, and 0.5% of total *P. acnes* proteins from the camel whey were dissolved in H_2O, as a positive control, which was also diluted at 1:1 in PBS. The mixtures were incubated in a water bath at 56 ^{o}C for 10 minutes, and then cooled

down in the refrigerator for 10 minutes. Afterwards, the mixtures were centrifuged at 13400 rpm for 10 minutes using microfuge. The aqueous phase (lower one) was collected using 1 ml syringe (about 400 µl).

2.2.5.3 Enzyme-Linked Immunosorbent Assay (ELISA) for the Cream Extract

Pre-coated and blocked ELISA plate (coated with 5 µg/mL of the purified *P. acnes* OMPs, and blocked with 2% Bovine Serum Albumin (BSA) in PBS) was incubated with 100 µL/well (as duplicates) from the stock and diluted cream extracted samples for one hour at room temperature with shaking. The plate was washed 3 times with 100 µL of 0.15 M PBS (containing 0.05% Tween 20), and blocked with PBS-T, 225 µL/well. Then, it was incubated with mouse anti-Camel IgG Abs, 100 µL/ well for one hour at room temperature with shaking. The mouse anti-camel IgG Antibodies source was from Pr. Gat D2F6 culture 1.8 mg\mL (1:4000), diluted in 1% BSA in PBS. After that, the plate was washed 3 times with PBS-T, 225 µL/well and then it was incubated with the secondary antibody (Goat anti-Mouse IgGs-HRP, diluted at 1:1000 in 1% BSA/PBS, 100 µL/well) for one hour at room temperature with shaking. Finally, the plate was washed 3 times with PBS-T, 250 µL/well, and the OPD substrate (1 mg/mL OPD in Citrate Buffer, 100 µL/well) was added and incubated for 5 minutes and then the plate was measured at 450 nm using the ELISA reader.

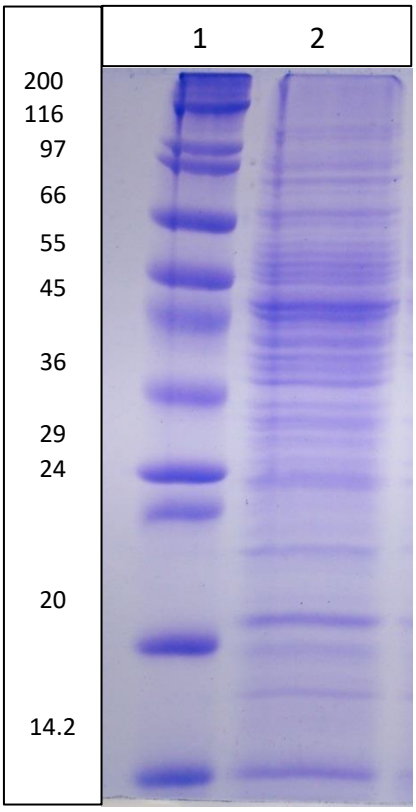
2.2.5.4 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) for the Cream Extract

The cream extract components were separated by the standard SDS-PAGE method. Briefly, 0.5 mm thick 10% acrylamidebisacrylamide gels under non-reducing conditions as were used. Resolving gels of 4 mL distilled water, 2.5 mL running buffer Tetramethylethylenediamine (TEMED) were firstly prepared. Stacking gels (4%) were prepared by adding 6 mL of distilled water, 2.5 mL stacking buffer (pH 6.6), 1.3 mL of 30% acrylamide-bisacrylamide solution, 100 µL of 10% Ammonium persulfate (APS), and 10 µL TEMED. The cream extract was mixed with an equal volume of sample buffer lacking β-mercaptoethanol (pH 6.8). Molecular weight protein standard ladder was used for estimation of band sizes. Electrophoresis conditions included running buffer with pH 8.3, 150 volts for 90 minutes, the loading volume was 20 µL/well. The gels were visualized after staining with 0.2% Coomassie brilliant blue R-250 and washing with 20% acetic acid until clear bands were seen.

Chapter 3: Production of Anti-*P. acnes* Antibodies

3.1 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of extracted *P. acnes* OMPs

SDS-PAGE is an important technique for molecular weight estimation of proteins. There are various protocols for proteins SDS electrophoresis, which differ in gel permeability depending on the size of the proteins to be separated and differ in buffer composition. All variations of the SDS-PAGE procedure are based on the ability of SDS to complex with proteins. Due to the presence of a twelve-carbon tail, SDS associates with non-polar regions of the protein molecule through hydrophobic interactions, while the polar head of the SDS molecule gives a net negative charge to the SDS–protein complexes.



Lane	1	2
Fraction	Wide Range Sigma Marker (kDa)	*P. acnes* OMPs extract

Figure 3.1: SDS-PAGE result for the extracted *P. acnes* OMPs

SDS-PAGE was carried out to map the Outer Membrane Proteins (OMPs) profile obtained from heat-killed *P. acnes*. As seen in the gel electrophoresis in Figure 3.1, the OMPs were loaded next to Sigma Marker™ Wide Range for comparing the location of OMPs with the standard ladder.

3.2 Bradford Protein Analysis of Extracted *P. acnes* OMPs

The concentration of the OMPs was determined using Bradford Colorimetric Protein Assay. Bradford protein analysis is used to measure the concentration of total protein in a sample and rely on the absorbance of the dye Coomassie Brilliant Blue G-250. The principle of this assay is that under acidic conditions, the red form of the dye is converted into its blue form, binding to the protein being assayed. If there's no protein to bind, then the solution will remain brown. The dye forms strong, noncovalent complex with the protein's carboxyl group by van der Waals force and amino group through electrostatic interactions. During the formation of this complex, the red form of Coomassie dye first donates its free electron to the ionizable groups on the protein, which causes a disruption of the protein's native state, consequently, exposing its hydrophobic sites (Bradford, 1976). The binding of the protein stabilizes the blue form of the Coomassie dye; thus the amount of the complex present in solution is a measure for the protein concentration, and can be estimated by use of an absorbance reading at 595 nm.

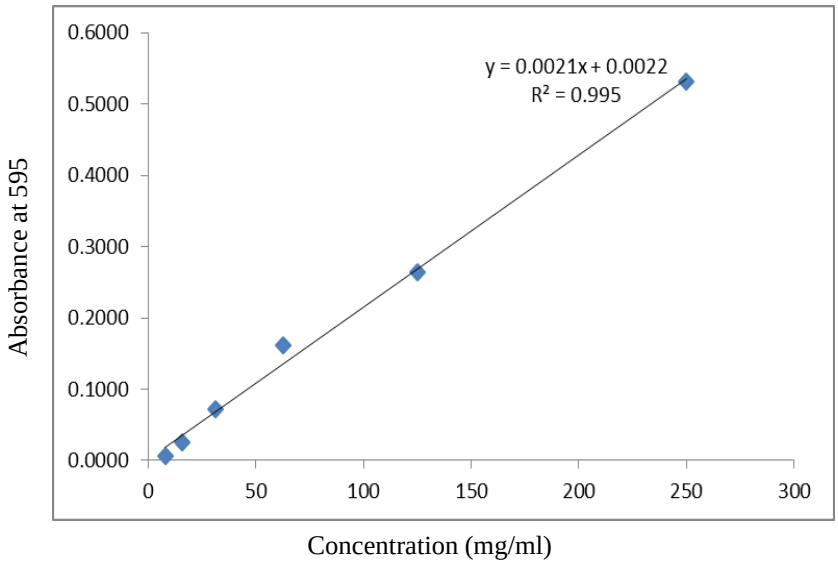


Figure 3.2: Bradford Protein Analysis Standard Curve for extracted *P. acnes* OMPs

Bradford protein analysis for the extracted *P. acnes*-(OMPs) was performed and the standard curve is shown in Figure 3.2. The concentration of the OMPs for this experiment was equal to 1367 µg/ml. Several optimization steps were conducted to determine the concentration of OMPs sufficient to induce a significant immune response in the female lactating camels.

3.3 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) for Camel Whey Enriched with Anti-*P. acnes* Antibodies

After the qualitative and quantitative analysis of the OMPs, the vaccine was injected into the female lactating camels and the milk was collected in timely manner. SDS-PAGE assay was carried out to map or confirm the existence of the anti-*P. acnes* antibodies in Camel whey after the immunization of the Camels.

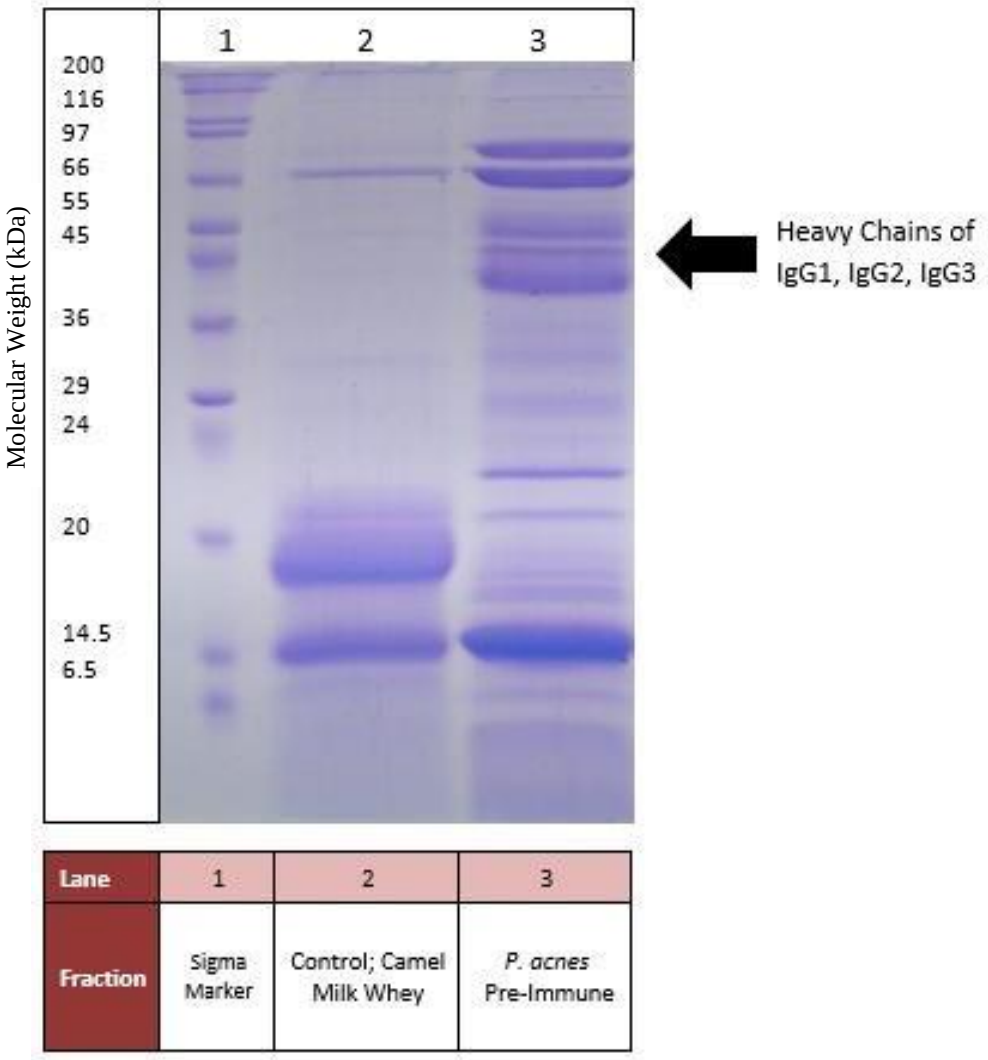


Figure 3.3: SDS-PAGE result for the Camel whey enriched with anti-*P. acnes* antibodies

The antibodies appeared at a molecular weight range of 35-65 kDa when compared to the Sigma Marker™ Wide Range, as shown in Figure 3.3.

3.4 Enzyme-Linked Immunosorbent Assay (ELISA) of Anti-*P. acnes* Antibodies in Camel Milk Whey against *P. acnes*

Camel Milk Whey was collected in timely manner and tested by ELISA (Figure 3.4).

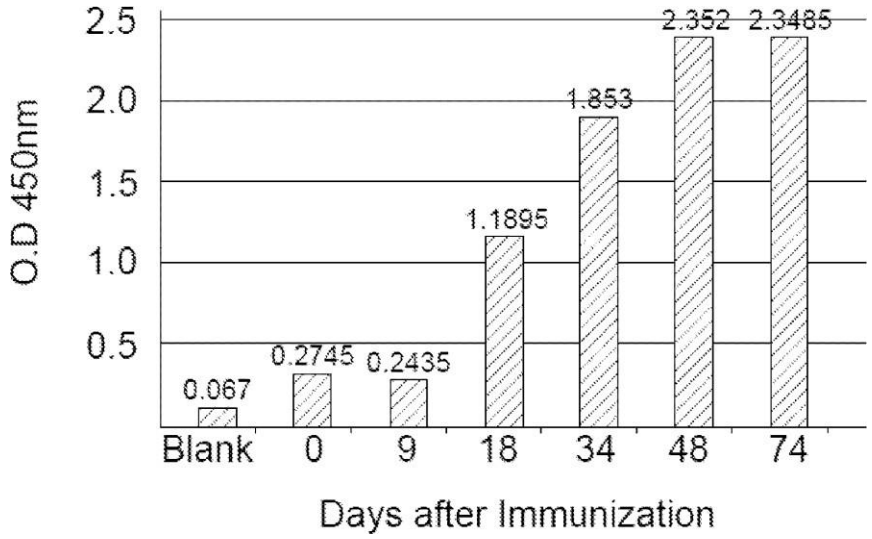


Figure 3.4: Enzyme-Linked Immunosorbent Assay of Camel Milk Whey enriched with anti-*P. acnes* antibodies against *P. acnes* and the Response over the Experiment Period

The results of the Enzyme-Linked Immunosorbent Assay (ELISA) of the anti-*P. acnes* antibodies against *P. acnes* showed that the concentration of antibodies increased successively with time (from day 0 to day 74), as seen in Figure 3.4. Interestingly, the response at day 18 has been more than four times the response at day 9. Several optimization steps were conducted to determine the concentration of OMPs sufficient to induce a significant immune response in the female lactating

camels, this optimization was guided by ELISA of the immunized camel milk whey against *P. acnes* antigens. One of the major and unique components of camel milk whey is the antibodies. The antibody fractions are composed of the conventional antibodies (IgG1, IgG2 and IgG3 subclasses) with the two heavy chains and two light chains Y shaped structures (Konnig *et al.*, 2017). However, it was also revealed that the unique and biologically active single chain antibodies (HCAb) are present in the whey collected in our studies with considerable quantities.

Chapter 4: Characterization of Camel Whey Proteins and Antibodies

In this study, it has been clearly shown that some protein fractions, which cannot be found in other animal species, are considerably present in the camel whey, such as; PGRPs. Interestingly, Casein, which is the major protein found in cheese and not in whey, does not compose high quantities in Camel milk collected in this study. In addition, camel casein has been previously proven to be less allergic than casein of cows (Pauciullo *et al*., 2014). This is very important since whey protein isolates used in our study are not 100% free of casein which would not have significant effects on the results obtained in our analyses for the evaluation of biological activities. The subsequent down-stream design for the adopted experimental purification and collection method of the biological protein fractions of camel milk whey was based on protein fraction molecular size, method specificity and availability of the chromatographic device. Protein G and Protein A Chromatographic separation was used to collect polyclonal antibodies. Validation of their purification was done using SDS-PAGE assay as shown in Figure 4.4. For purposes of method specificity, Heparin LP-LC chromatography was used to collect Lactoferrin and PGRPs, in spite of their appearance on the SDS-PAGE gel representing the protein fraction collected by protein G and A method. Lactoferrin and PGRPs presence was validated using SDS-PAGE assay, however, silver stain was used in case of PGRP.

4.1 Camel Whey Proteins Separation by Heparin Affinity Chromatography

Affinity Chromatography (AC). AC separates proteins using bio-recognition. The separation is based on the reversible interaction between the protein and a specific ligand attached to a chromatographic matrix. The sample is applied under conditions that favor specific binding to the ligand, while the unbound material is washed away. The high selectivity of affinity chromatography is caused by allowing the desired molecule to interact with the stationary phase and be bound within the column in order to be separated from the undesired material which will not interact and elute first. The molecules no longer needed are first washed away with a buffer while the desired proteins are let go in the presence of the eluting solvent (of higher salt concentration). This process creates a competitive interaction between the desired protein and the immobilized stationary molecules, which eventually lets the now highly purified proteins be released (Schlichthaerle et al., 2019; Sjobring et al., 1991).

The absorbance results of the peaks that resulted from the Heparin Affinity Chromatography separation of the Camel whey proteins are reported in Table 4.1.

Table 4.1: The absorbance results for the eluted peaks from the Heparin Affinity Chromatography separation

Peaks	Absorbance
Maximum Wash Peak	0.7036
Maximum Elution Peak (Peak 1)	0.0059
Maximum Elution Peak with 2 M NaCl	0.3383

4.1.1 Validation of the Results of Heparin Affinity Chromatography

The results obtained from Camel whey proteins separation using Heparin Affinity Chromatography were validated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) with Coomassie stain for Lactoferrin, as shown in figures 4.1 and 4.2, and with silver staining for Peptidoglycan Recognition Proteins (PGRPs), as shown in Figure 4.3.

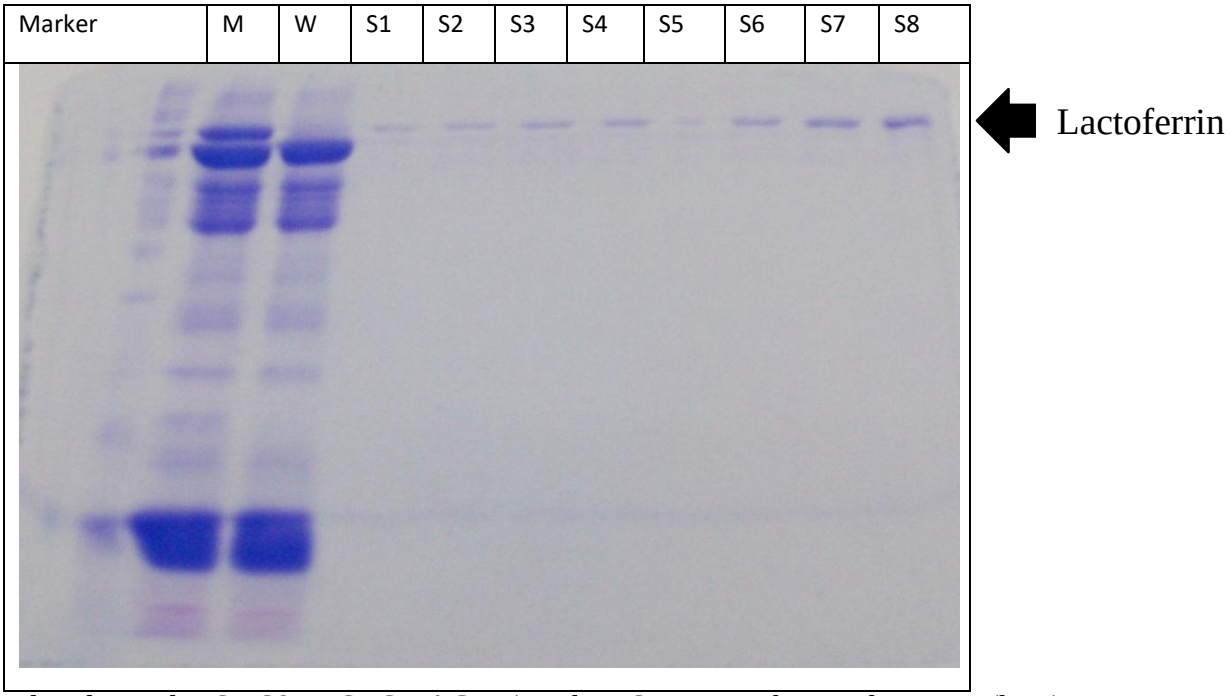


Figure 4.1: Eluted samples S1-S8 on SDS-PAGE. (Marker: Sigma Marker Wide Range (kDa), M: Milk sample, W: Wash)

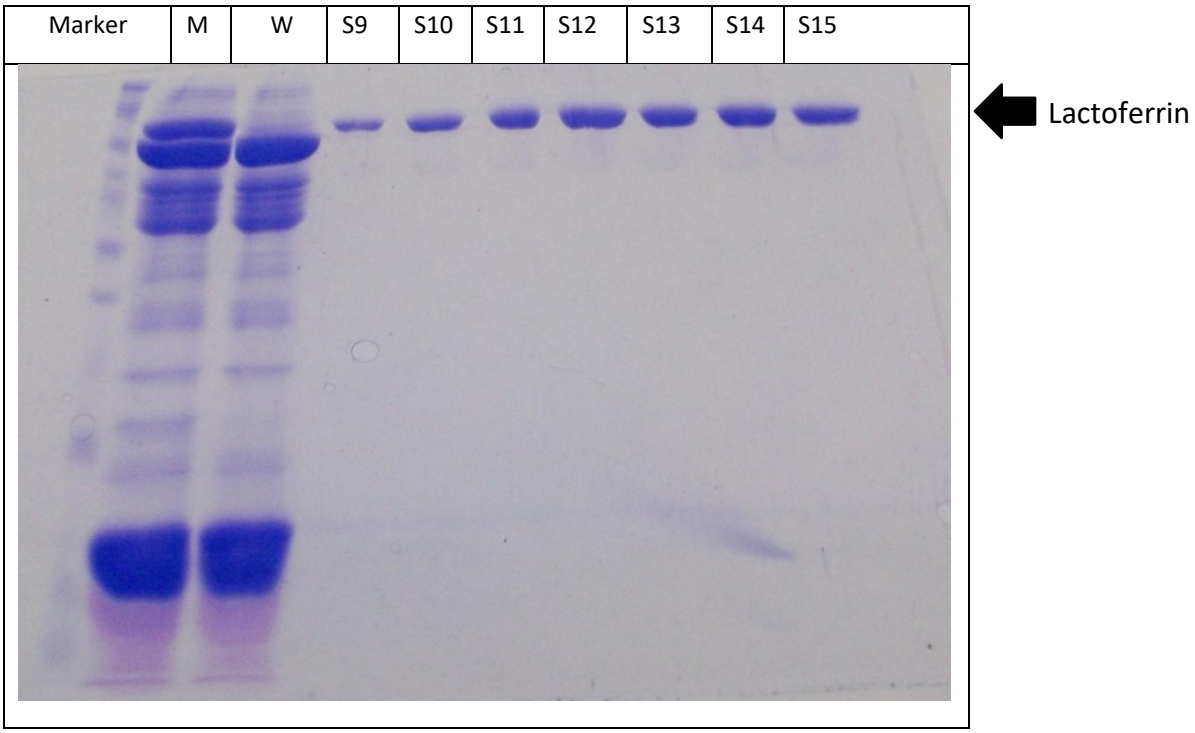


Figure 4.2: Eluted samples S9-S15 on SDS-PAGE. (Marker: Sigma Marker Wide Range (kDa), M: Milk sample, W: Wash)

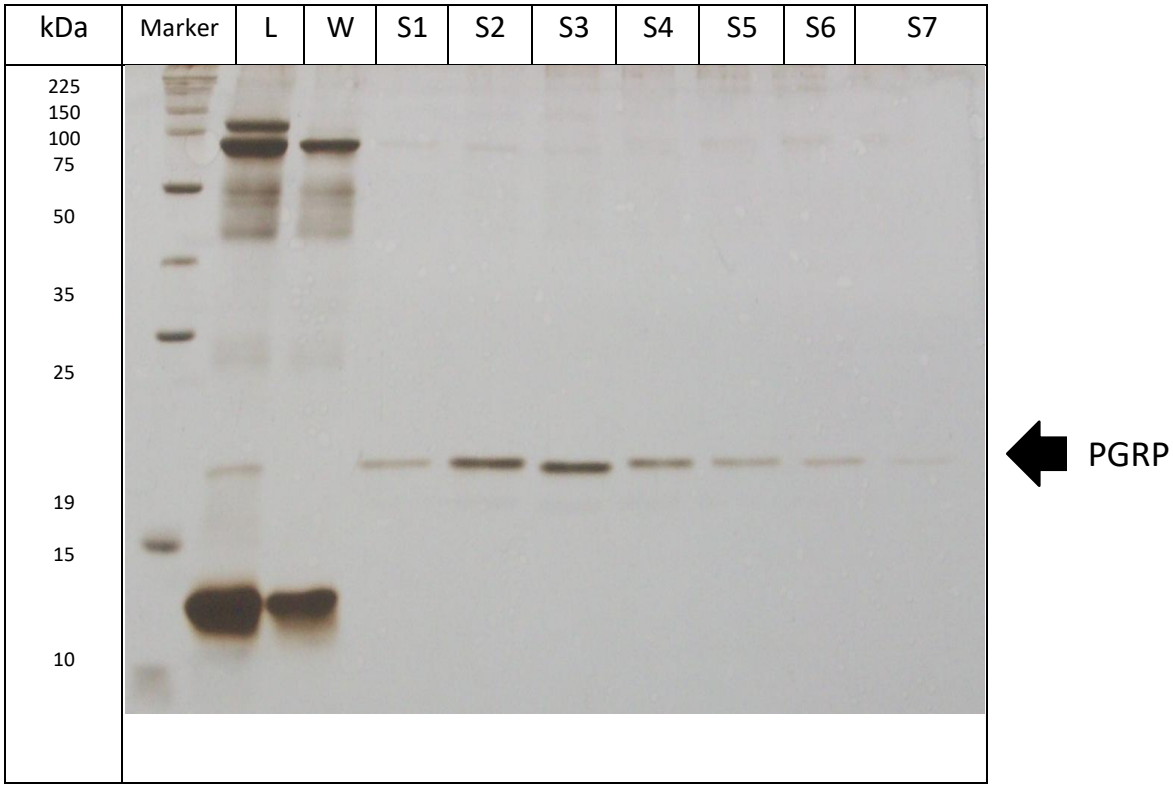


Figure 4.3: Eluted samples S1-S7 on SDS-PAGE. (Marker: Broad Range Protein Molecular Weight Marker, L: Load, W: Wash)

4.2 Camel Whey Proteins Separation by Protein A and Protein G Chromatography

Protein G and Protein A Chromatographic separation was used to collect polyclonal antibodies. Protein G is an immunoglobulin-binding protein found in Streptococcal bacteria much like Protein A but with differing binding specificities. They are cell surface proteins that are used in the application of purifying antibodies immobilize

or detect immunoglobulins. Each of these immunoglobulin-binding proteins has a different antibody binding profile in terms of the portion of the antibody that is recognized and the species and type of antibodies it will bind to. Protein A/G is a recombinant fusion protein that possesses IgG binding domains of both proteins. Protein A/G can bind to all subclasses of human IgG, making it useful for purifying polyclonal and monoclonal antibodies and others whose subclasses have not been determined (Sikkema, 1989).

Table 4.2: The absorbance for the eluted peaks from the Protein G and Protein A Chromatography separation

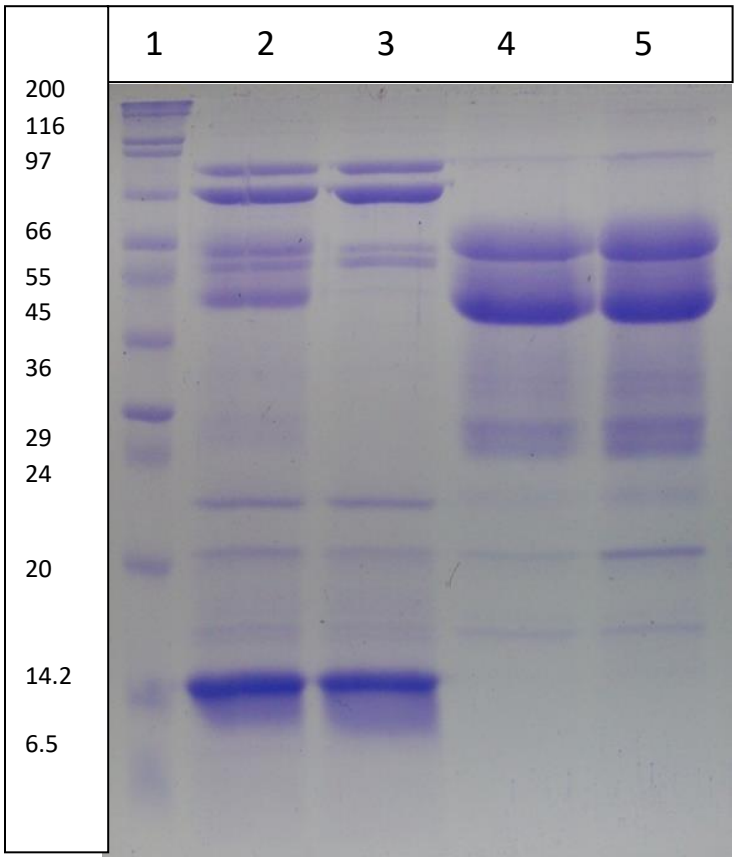
Peaks	Absorbance
Maximum Wash Peak	0.25
Maximum Elution Peak	0.54

The absorbance results of the peaks that resulted from the Protein G and Protein A Chromatography separation of the Camel whey proteins are reported in Table 4.2.

4.2.1 Validation of the Results of Protein G and Protein A Chromatography

The results obtained from Camel whey proteins separation using Protein G and Protein A Chromatography were validated by Sodium Dodecyl Sulfate-

Polyacrylamide Gel Electrophoresis (SDS-PAGE) with Coomassie stain for, as shown in Figure 4.4.



Lane	1	2	3	4	5
Fraction	SDS Marker (kDa)	Loaded Whey	Wash peak	Elution Peak	Elution Peak

Figure 4.4: Coomassie stained SDS-PAGE for the purified immunoglobulins after Protein G and Protein A Chromatography

Chapter 5: Validation (*In vitro* and *In vivo* examination)

5.1 In Vitro Testing of the Antimicrobial and Anti-inflammatory Efficacy of Immunized Camel Whey against Propionibacterium acnes

5.1.1 Microbiological Examination

Camel whey contains many components that are reported to have antibacterial effects against gram-positive and gram-negative bacteria. It has been described that lysozyme, lactoferrin and PGRPs can inhibit bacterial growth (El Sayed *et al.*, 1992, Kappeler *et al*., 2004). Lactoferrin for example inhibited *Salmonella typhimurium* growth by binding to iron, which is essential for bacterial growth, in the surroundings, thus inducing a bacteriostatic effect (Ochoa 2009). In contrast, lysozyme has been described to cause some damage to the bacterial cell wall (Konuspayeva *et al*., 2007, Benkerroum 2008).

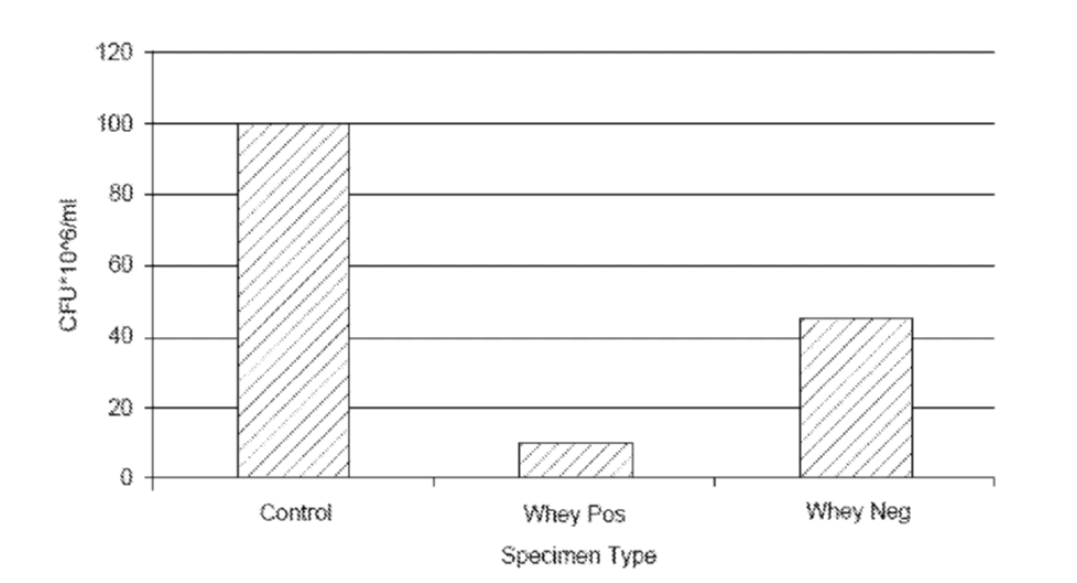


Figure 5.1: Bactericidal activity of immunized Camel whey against *P. acnes* incubated for 72 hours

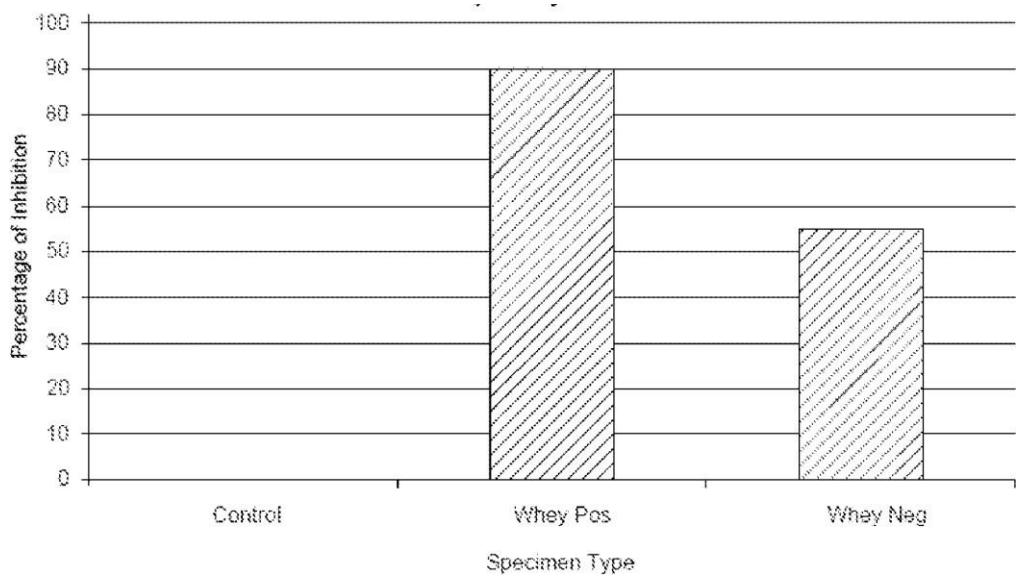


Figure 5.2: The percent of growth inhibition of immunized Camel whey against *P. acnes* incubated for 72 hours

A standard inoculum of *P. acnes* was challenged by mixing it with immunized Camel whey, and colony forming units (CFUs) were counted after incubation for 72 hours. The results obtained were compared with a standard inoculum devoid of the immunized Camel whey used as negative control. The results obtained show significant reduction in the colony forming units when the immunized Camel whey was used compared to the standard inoculum (negative), as seen in Figure 5.1. The percentage of growth inhibition of *P. acnes* was determined, as shown in Figure 5.2; immunized Camel milk inhibited the growth of 90% of the colony forming units compared to the negative control.

5.1.2 Agar Microdilution Assay of Antimicrobial Peptides (AMPs) against *P. acnes*

The antimicrobial activity of the isolated Camel whey derived proteins; Lactoferrin, Peptidoglycan Recognition Proteins (PGRPs), and anti-*P. acnes* antibodies, was evaluated using the agar microdilution assay.

Table 5.1: The growth inhibitory effect of Lactoferrin, PGRPs, and Anti-*P. acnes* antibodies against *P. acnes*

Protein Preparation	ID_{90} (mg/mL)
PGRPs	0.2
Lactoferrin	0.8
Anti-*P. acnes* Antibodies	NE
Whey Protein Isolate	0.8

(ID_{90}; Inhibitory concentration needed to inhibit the growth of 90% of the bacteria, NE: No Effect)

The results obtained show that the highest antimicrobial activity against *P. acnes* can be attributed to PGRPs and similar between complete milk whey proteins and Lactoferrin. Anti-*P. acnes* antibodies didn't show growth inhibitory or bactericidal activity against *P. acnes* as seen in Table 5.1.

In harmonization with our results and based on previous studies, the antibody fractions have the least antimicrobial activity in natural camel whey. The antibody fractions isolated from Camel whey did not show anti-bacterial activities. This result can be attributed to the biological mechanisms of antibodies which are working mainly as opsonins and complement fixing proteins so by themselves are not effective as bactericidal molecules. The only described effect of these antibodies is their neutralizing effect against toxins and soluble factors produced by pathogenic bacteria and viruses (Ochoa *et al*., 2009)

Some studies elucidated the major role of α-lactoalbumin as the most active components of camel whey proteins that can protect newborns from infection with different pathogens (Benkerroum 2004, Almajali *et al*., 2007). Indeed, bactericidal activity could not be described solely in vitro for the tested Camel whey protein isolate and even for each protein fraction separately. Nevertheless, the growth inhibitory effects against gastrointestinal pathogens like *E. coli* and *Samonella* species for example would aid in the management of infections caused by these pathogens providing that the heat and acid stability of whey proteins described above provided an added value for therapeutic considerations (Jrad et al., 2014).

One of the major supporting evidence to our results of the low anti-bacterial effects of natural camel whey (not the hyper-immunized) is described in a study that used pepsin and pancreatin to digest camel colostral whey proteins into peptides. The colostral whey protein still exhibited antibacterial activity against Gram-negative and Gram-positive bacteria after enzymatic digestion with a remarkable enhancement of other biological activities such as antioxidant activity and ACE inhibition (Salami *et al*., 2010). The release of the bioactive peptides from the hydrolysate of whey protein particularly lactoferrin was responsible for the stronger antibacterial activity of the colostrum whey peptides compared to the native LF

emphasizing the fact that chemical, biological and physical treatment of the camel whey are required to improve its efficacy as antimicrobial agent (Almajali *et al*., 2009).

5.1.3 Pro-Inflammatory Cytokines Analysis

Analysis of the inhibitory activity of Camel whey derived Antimicrobial Peptides (AMPs) on pro-inflammatory mediators' secretion in co-cultures of THP-1 cells with heat-killed *P. acnes* was performed to further investigate the role of AMPs in inflammatory acne. The release of the pro-inflammatory mediators, particularly TNF-α and IL-8, was significantly reduced as seen in Figure 5.3, showing the Enzyme-Linked Immunosorbent Assay (ELISA) results.

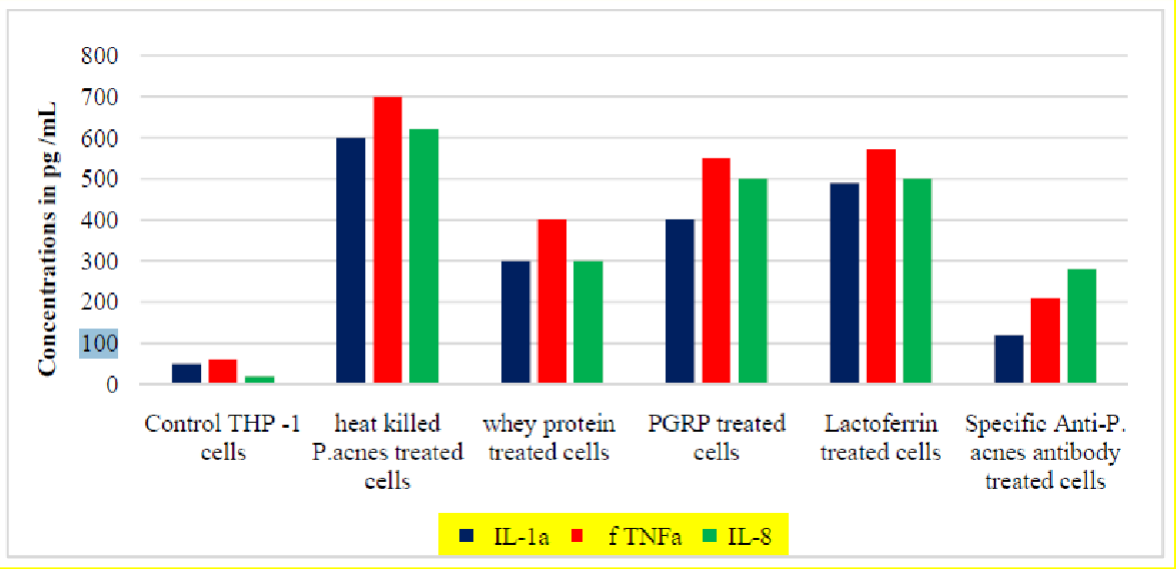


Figure 5.3: The effects of immune protein fractions of Camel milk on the release of pro-inflammatory cytokines TNF-α, IL-8 and IL-1α in THP-1 cells treated with heat-killed *P. acnes* (THP-1 not treated with heat-killed *P. acnes* were used as control)

The Anti-*P. acnes* antibodies isolate exhibited the greatest anti-inflammatory effect. After which, the complete whey protein exhibited significant anti-inflammatory effect suggesting the presence of an additive or synergistic effect of other AMPs or small molecules present in Camel milk. Lactoferrin and PGRP had an inferior effect to the release of pro-inflammatory mediators, subsequently, they would have no major role in the management of inflammatory acne especially if they were to be used solely.

In contrast to the weak anti-microbial activity of the immunized Camel whey antibodies, the results of this study support the evidence of potent anti-inflammatory

and immune modulating effects of native whey proteins isolated from Arabian camels. Similar to our results, anti-inflammatory and immune modulating effects of some whey proteins of camel were described both *in vitro* and *in vivo* models (Badr *et al*., 2013). The presence of high quantities of innate immune proteins in whey of camel milk specially PGRPs in addition to their modulating effects on immune cell functions, such as enhancing lymphocyte activation, proliferation and chemotaxis; cytokine secretion; antibody production; phagocytic activity; and granulocyte and NK cell activity, might be essentially responsible for this unique biological properties of camel whey (Badr *et al*., 2017). In particular, whey proteins enhance the production of IL-1α, IL-8, IL-6, macrophage inflammatory proteins (MIP-1α, MIP-1β), and tumor necrosis factor (TNF-α) (Rusu *et al*., 2010). In a study conducted on diabetic mice, whey proteins of camels have been shown to improve the immune response through modifying cytoskeletal rearrangements and chemotaxis in B and T cells (Badr *et al*., 2017). Furthermore, Whey peptides have immunomodulatory activities, such as stimulating lymphocytes and increasing phagocytosis and the secretion of IgA from Payer's patches (Beaulieu *et al*., 2006, Krissansen *et al*., 2007). The addition of whey proteins of camel to the diet is shown to significantly improve primary and secondary intestinal tract antibody responses to a variety of different vaccine antigens that are currently in medical use (Low *et*

al., 2003). As described previously, a diet containing 20% protein from camel whey proteins was able to significantly enhance the immune response to influenza vaccine, diphtheria and tetanus toxoids, poliomyelitis vaccine, ovalbumin, and cholera toxins and more importantly wound healing (Low *et al*., 2003, Badr et al., 2013). To sum up, the highest antimicrobial activity against *P. acnes* can be attributed to PGRPs and similar between complete milk whey proteins and Lactoferrin while the Anti-*P. acnes* antibodies did not show anti-bactericidal activity. However, the Anti-*P. acnes* antibodies exhibited the greatest anti-inflammatory effect, and the complete whey protein exhibited significant anti-inflammatory. Lactoferrin and PGRP had an inferior effect to the release of pro-inflammatory.

5.2 In Vivo Efficacy Testing for the Whey Protein Concentrate of the Milk of Immunized Camels against Propionibacterium acnes Using Rabbit Ear Model

The use of animals for scientific purposes is both a long-lasting practice in medicine and biological research. The remarkable physiological and anatomical similarities between animals and humans; particularly mammals, have prompted researchers to novel therapies in animal models before applying their discoveries to humans. The use of animals is not only based on the enormous commonalities in most mammal's biology, but also on the fact that human diseases often affect other animal species.

It is particularly the case for most infectious diseases but also for very common conditions such as Type I diabetes, hypertension, allergies, and so on. Not only diseases are shared, but also the mechanisms are often so similar that 90% of the drugs used in veterinary are identical or very similar to human's drugs. Major breakthroughs in medical research have been possible because of testing on animal models.

5.2.1 Gross Examination of Rabbit Ears

Rabbit Ear Model was used to assess the antimicrobial and anti-inflammatory effects of Camel whey enriched in anti-*P. acnes* antibodies against *P. acnes*. Gross examination of the rabbit ears showed double fold increase in the infection-induced rabbit ear compared to the normal non-infected rabbit ear. Upon the injection of Camel whey enriched with anti-*P. acnes* antibodies, ear thickness was significantly reduced, milder redness, heat, and no papules nor pus were seen as shown in Figure 5.4.

(a) Control	(b) Positive whey treated	(c) Negative whey treated	(d) Infection induced

Figure 5.4: Comparison of ear thickness, and the appearance of redness, heat, or formation of papules during rabbit ear gross examination. (a) Normal, (b) reduced thickening, milder redness and heat, no papules nor pus are reported, (c) less reduced thickness, moderate redness and heat, no papules nor pus are reported, (d) thickening, redness, heat and papules are typical signs of inflammatory response in *P. acnes*

One of this research objectives is evaluating the antimicrobial and anti-inflammatory effects of immunized camel milk whey and its components against *P. acnes.* The results of the in-vitro testing detailed above has shown that the highest antimicrobial activity against *P. acnes* can be attributed to PGRPs and Lactoferrin while the Anti-*P. acnes* antibodies did not show growth inhibitory or bactericidal activity against *P. acnes* as seen in Table 5.1. However, the whey protein isolate exhibited the greatest anti-inflammatory effect suggesting the presence of a synergistic effect of other AMPs or other small molecules present in Camel milk. These *in-vitro* findings are reflected on the rabbit *in-vivo* testing showing that rabbit ear thickness was

significantly reduced in cases of injecting pretreated bacteria with positive whey and less significantly in cases of negative whey.

5.2.2 Histopathological Examination of Rabbit Ears

The inflammatory response in the infection-induced rabbit ear was presented with dense mixed inflammatory cells, marked eosinophilia, and the formation of micro abscess and ulcerations without affecting the epidermis. On the other hand, upon injecting the infection-induced rabbit ear with Camel whey enriched with anti-*P. acnes* antibodies, mild mixed inflammatory cells with no ulceration and less micro abscesses were markedly observed. No difference was observed in the rabbit ear treated with the negative whey compared with the rabbit ear treated with positive Camel whey. This work has been done with the assistance of an expert in the field and the results of the histopathological examination are shown in Figure 5.5.

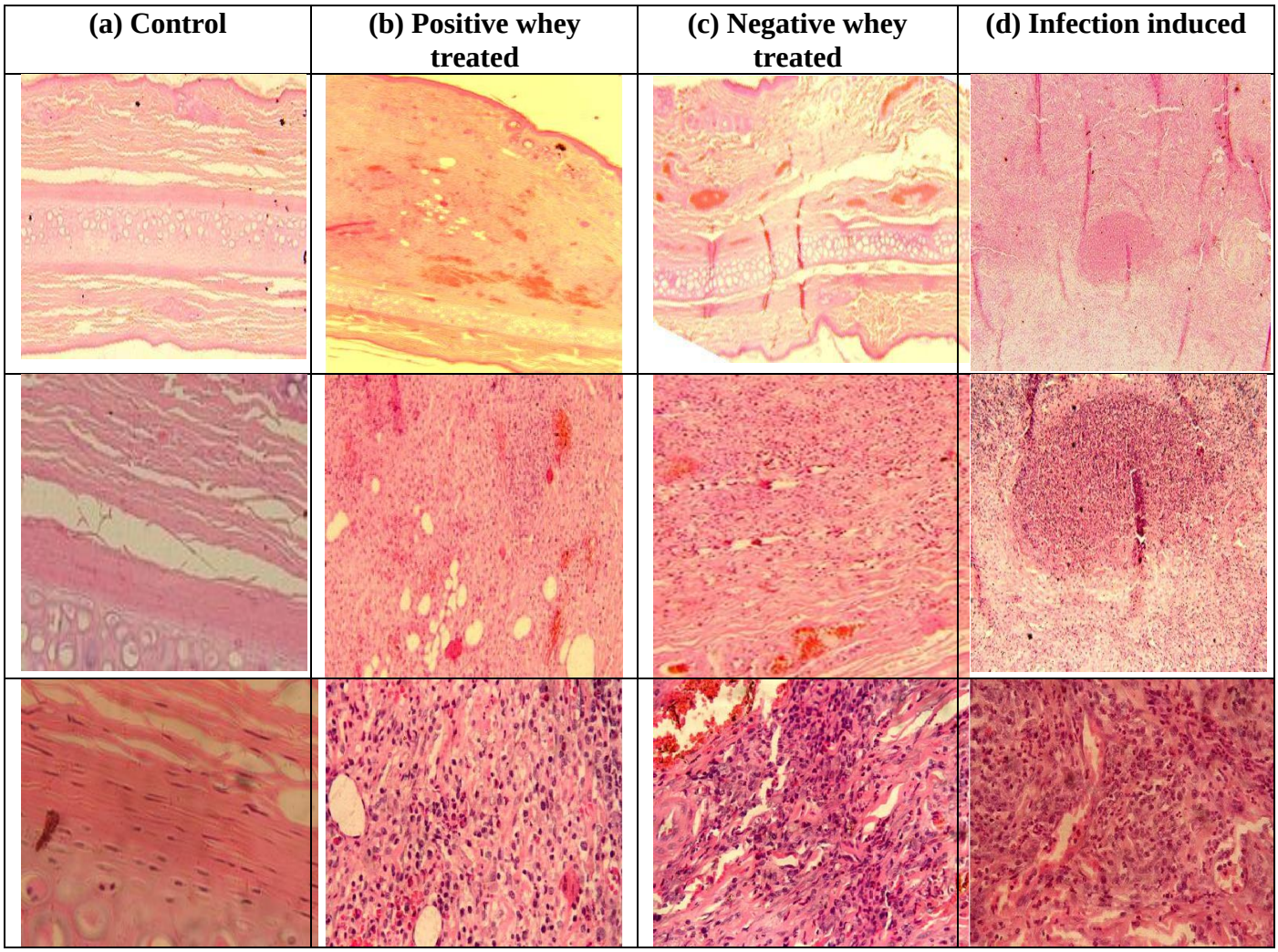


Figure 5.5: Comparison of histopathological changes in rabbit ear tissues after treatment with positive and negative camel whey. Positive and negative control groups were also used in the comparison.(a) Normal, (b) reduced thickening, milder inflammatory response, no ulceration and less to rare micro-abscesses are observed, (c) less reduced thickness with dense mixed inflammatory cells and ulceration are observed. Less micro-abscesses are less frequently reported, (d) dense mixed inflammatory cells with notable eosinophils in increased thickness or rabbit ear injected by *P. acnes*. In addition, micro-abscess are observed

Moreover, the histopathological examination of the infection induced rabbit revealed that the inflammatory response was presented with dense mixed inflammatory cells and marked eosinophilia, formation of micro abscess and ulcerations without affecting the epidermis. In contrast, mild mixed inflammatory cells without ulceration and less micro abscesses were markedly observed in cases of injecting pretreated bacteria with positive whey. No differences were observed in the negative whey treated rabbit in comparison to the infection induced ear.

The in-vivo examination is matching the in-vitro findings and supporting the immunized camel milk whey components has antimicrobial and anti-inflammatory against *P. acnes.*

Chapter 6: Formulation of Topical Anti-*P. acnes* Products

6.1 Description of the Topical Composition

Based on the conducted research, a novel pharmaceutical topical composition with the milk of immunized camel (*Camelus dromedarius*) as its main component (i.e. active ingredient) has been developed and patents have been filed, with the purpose of using the proposed composition for the topical treatment and/or prevention of *Acne Vulgaris*. The invention described also reveals a method for preparing the stated composition.

Four topical formulations have been developed based on this invention. The finalized formulae are described below:

1) Facial Wash:

A gentle cleanser for acne prone skin that cleanses while preventing breakouts. The facial wash has been formulated to be devoid of any harsh ingredients that can potentially dry out the skin's natural oils, which leads to oil overproduction on the skin surface which can ultimately lead to more acne. Its major function is to cleanse and remove oil, dirt, and dead skin without drying the skin in the process.

2) Serum:

A lightweight serum that has been formulated to be easily absorbed by the human skin and to reach deep into its layers. It targets the acne spots specifically and has the richest content of antibodies compared to the rest of the other topical formulations.

3) Cream:

A cream that addresses acne-causing bacteria while simultaneously moisturizing the skin. It contains 3.5% camel whey, and aids in acne treatment and spots and blemishes reduction while maintaining the skin's freshness. It has been formulated to suit normal to dry skin as well as sensitive skin.

4) Gel:

A gel formula that has a non-greasy texture and addresses the acne-causing bacteria. It is formulated to accelerate the removal of acne while maintaining the hydration of the skin.

All of these topical formulae rely on AMPs derived from camel milk whey as their active ingredient. This invention is considered the first patented bio-based skincare innovation developed from camel whey-derived antibodies.

6.2 Preparing a Pilot for the Cream Formula 0.5 w/w%

A pilot formula for the topical cream has been developed to prepare an initial sample at the laboratory. The camel whey concentration aimed for was 0.5 w/w%, which was proven to be effective during the in vivo studies on rabbit ears. Table 3.4 shows the formula used to prepare the cream.

Table 6.1: Pilot Cream Formula 0.5 w/w%

Material	Weight	mg/unit
Dried Camel Whey*	15g	2.5
Cetyl Alcohol	90g	15
Tween-20	30.9mL	5.15
Glyceryl Monostearate Type1	17.1g	2.85
MERKUR 791	60g	10
Purified Water	387mL	64.5
Total	600	100

*Equivalent to 0.5 w/w% (protein/cream)

6.2.1 Anti-*P. acnes* Antibodies Activity Assessment in the Cream Formula

The percentage of activity for the anti-*P. acnes* antibodies in the pilot cream formula was evaluated to ensure their stability at room temperature. The percentage of activity for the cream formula was calculated by dividing the readings of samples on

the readings of purified antibodies stored at -80^{0}C. The results are shown in figure 6.1. The antibodies in the cream formula retained around 75% of their activity after 8 weeks of storage at room temperature.

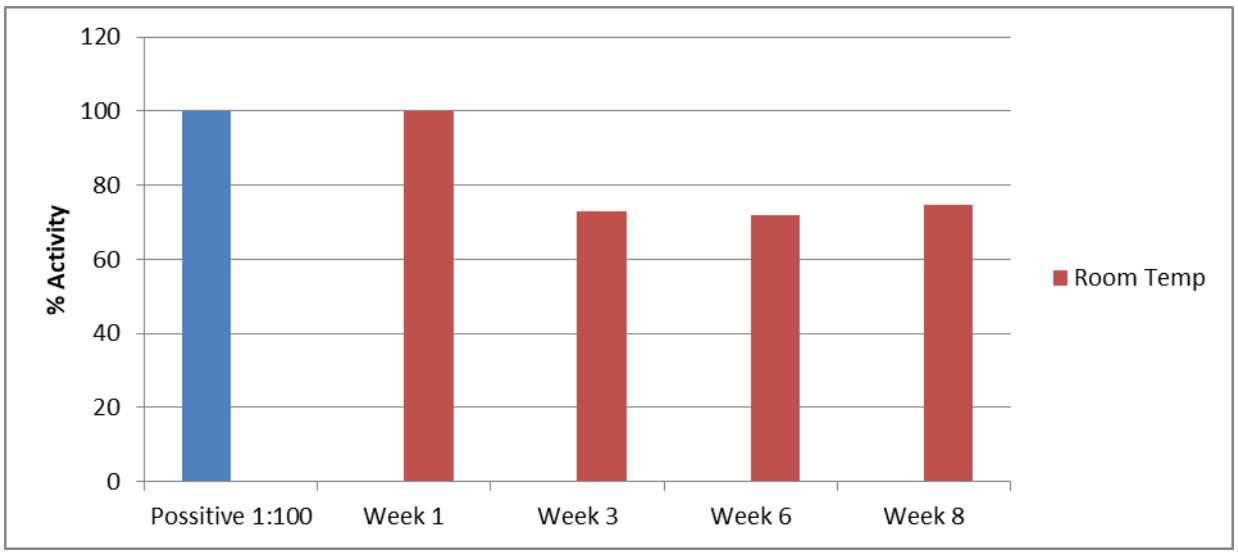
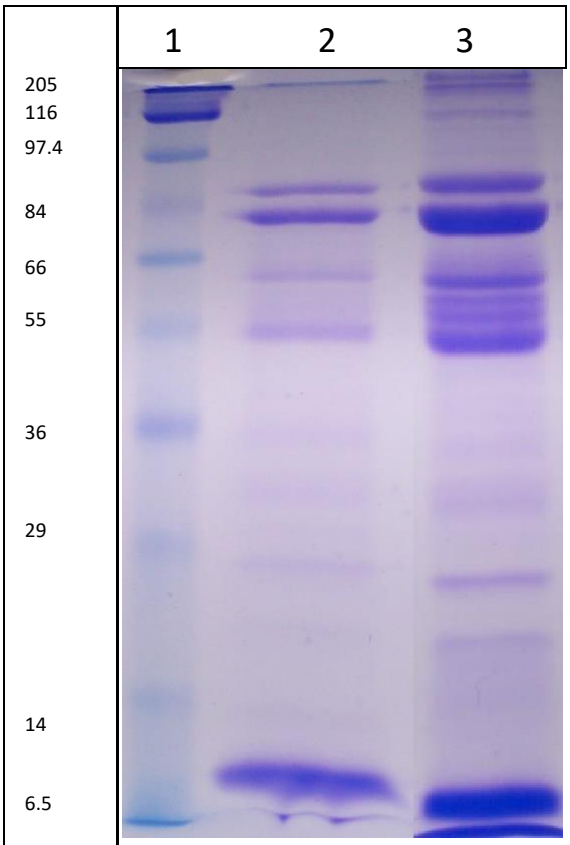


Figure 6.1: The stability of the anti-*P. acnes* antibodies in the cream formula after 8 weeks of storage at room temperature

6.2.2 Anti-*P. acnes* Antibodies Profile in the Cream Extract

The anti-*P. acnes* antibodies profile for the cream extract was mapped using SDS-PAGE as shown in Figure 6.2.



Lane	1	2	3
Fraction	Sigma Marker (kDa)	R.T cream extract	Positive *P. acne* whey

Figure 6.2: Anti-*P. acnes* antibodies in the cream extract after 8 weeks of storage at room temperature

The second objective of this research is to transfer our findings from bench to market; which is through developing a pharmaceutical formula enriched with the hyper-immunized camel milk whey that harnesses all its beneficial properties against Acne vulgaris. Thereof, novel pharmaceutical topical composition formulae enriched with the milk of immunized camel (i.e. active ingredient) have been developed and patents have been filed with the purpose of using the proposed products for the topical treatment and/or prevention of Acne Vulgaris, including cream, gel facial wash and serum. As an example, we have presented above in section 3.4.2, the prepared Pilot Cream Formula 0.5 w/w%. This pilot formula for the topical cream has been developed to prepare an initial sample at the laboratory. The camel whey concentration was 0.5 w/w%. To validate the stability of the topical cream, a stability study for opened cream was conducted after being stored for 8 weeks at room temperature. The antibodies in the cream formula retained around 75% of their activity after those 8 weeks, and the stability has been validated using SDS-PAGE, which confirmed the presence of AMPs and efficiency of the protein purification and its utilization in topical cream preparation.

Chapter 7: Conclusion and Future Work

7.1 Conclusion

Despite native camel whey proteins intrinsically having acceptable biological activities, the concept of enriching the camel whey proteins with extra biological molecules with unique specificity and better potency has been established as the major intended outcome of this work. This study designed a vaccination based platform of Camels containing antigens for bacterial infections which have in addition to the pathogenic effects of the bacterial etiologic agent, an inflammatory response based pathogenesis. We selected Acne Vulgaris as a typical example for this purpose. The resulted whey here is now known as hyper-immune whey shall contain beside the native whey biologically active proteins, extra-molecules and proteins like specific antibodies and other pro-inflammatory or anti-inflammatory molecules which will theoretically be responsible for the added effect of the tested Camel whey. In spite of the low relative abundance of immunoglobulins in camel milk, hyper-immune whey would have higher contents of antibodies.

Hyper-immune whey and milk have been extensively studied in the literature for both dietary and health benefits (Badr *et al*., 2017). Currently, there are many companies that produce commercial hyper-immune whey. Immunization of cows with different types of antigens derived from parasitic, bacterial and viral agents and

the subsequent purification of polyclonal and monospecific antibodies for preventive and treatment therapies has been reported (Hurley 2011). As part of the passive immune therapy, hyper-immune whey of cows clinically proved to cure patients from Rota virus however, since the immunoglobulins of cow milk are acid labile, their utility in the clinical practice is limited to the management of gastrointestinal pathogens.

In this manner and to the best of our knowledge, this is the first study which produced hyper-immune whey from camel milk and characterize its biological activities. Indeed, as previously proven, camel proteins have better chemical and physical stability compared to proteins from other mammalian species. Moreover, the less allergic potential of camel proteins would add a value of hyper-immune whey as a supportive and/or alternative therapeutic potential for many disorders and diseases.

Acne is one of the most complicated disorders affecting adults worldwide. Due to the combined pathogenic and immunological pathophysiology associated with this disorder, different therapeutic options can be postulated for the management and improvement of susceptible individuals. In this study, we derived and vaccinated camels with heat killed antigens of *P. acnes* and collected the so called hyper-immune whey. At the first step, the whey was characterized for its major protein

contents and then for the presence of anti-*P. acnes* antibodies which subsequently were purified. Then we compared the biological effects of the hyper-immune whey protein fraction to the antibody fraction against *P. acnes* and its associated inflammatory potentials. Indeed, the presence of cross-reacting antibodies in the native whey could not be excluded and might be attributed to some bacterial flora present in the camels which could not be investigated in this study.

In this study we evaluated the antimicrobial and anti-inflammatory activity of camel whey derived and the whole whey components against acne models in vitro. In specific, we analyzed the activity of Lactoferrin, peptidoglycan recognition proteins (PGRPs) and immunoglobulins specific to *P. acnes*. These antimicrobial proteins were selected because of their abundancy, stability and known superior physiochemical and biological activities making them excellent potential for use in prophylactic and therapeutic cosmeceutical formulas (Al-Qaoud *et al*., 2014). The antimicrobial effect of the tested protein factions by the microdilution assay revealed highest activity of PGRPs against *P. acnes* while *Anti-P. acnes* specific antibodies did not show growth inhibitory effects or bactericidal activity against *P. acnes* when tested as a separated fraction. Interestingly, the hyper-immune whey showed high efficacy as anti-acne compared to all of the separately tested protein fractions. It

was clear that PGRPs Inhibited the growth of *P. acnes* more efficiently than other protein fractions isolated from camel milk.

To further investigate whether these selected protein fractions possess biological properties against inflammatory acne, analysis of their inhibitory effects on the secretion of pro-inflammatory mediators in co-culture of THP-1 cells with heat-killed *P. acnes* was conducted. It was previously described that *P. acnes* is a major factor that is involved in the inflammatory nature of acne since the ability of this micro-organism to metabolize fatty acids in the sebum and the production of extra-cellular toxins like CAMP factor would induce monocytes to secrete pro-inflammatory cytokines especially TNF-α, IL-1α, and IL-8 (Kim, 2005). We performed an ELISA for TNF-α, IL-1α, and IL-8, in supernatants THP-1 monocytes of treated with heat killed *P. acnes*. The specific antibodies against *P. acnes* reduced significantly the release of the pro-inflammatory mediators specially TNF-α and IL-8. In contrast, PGRPs and Lactoferrin were inferior as inhibitors to the release of these pro-inflammatory mediators and they would have no major role in treating inflammatory acne particularly if they will be used individually not in combination based therapeutic regimens. Interestingly, the best revealed anti-inflammatory effect successively after the *P. acnes* antibodies was seen in the hyper-immune whey

protein fraction indicating the presence of potential synergism or adding effect of other molecules in camel milk. TNFα is a multi-effector cytokine produced mainly by activated macrophages. IL-8 is a major cytokine in the inflammatory acne process in which it works as a chemotactic factor, which promotes attraction of neutrophils to the pilosebaceous unit (Kurokawa *et al*., 2009).

Numerous studies have addressed the distinctive biological activities of camel milk in different infections and diseases management. Concerning the antimicrobial activity of camel milk, potent anti-Staphylococcal and anti-listeriosis activities of colostrum collected from camel milk were reported (Benkerroum 2010, Ng *et al*., 2015). Moreover, anti-cancer and anti-inflammatory activities of camel milk have been reported (Abu-Qatouseh et al., 2019). However, the underlying mechanisms behind these biological effects are still under investigation. Here, we report for the first time the anti-acne potential of PGRPs isolated from immunized camel milk whey with a modest anti-inflammatory activity. The benefit of camelid PGRPs is their long term stability and wide spectrum of activity. PGRPs are part of the pathogen associated molecular pattern (PAMPs) molecules which are essential components of the innate immune system (Liu et al., 2000). According to Sharma et al., 2011, camelid PGRPs have distinct structural moiety compared to human

PGRPs and these moieties allow camelid PGRPs to bind to LPS of Gram negative bacteria, Lipoteichoic acid of Gram positive bacteria in addition to the peptidoglycan layer of bacteria (Sharma *et al*., 2011). Few reports have shown anti-inflammatory effects of human PGRPs. In a study conducted by Park *et a*l., 2011, PGRPs expressed mice skin have modulated the sensitivity to experimentally-induced atopic dermatitis and contact dermatitis. Camel antibodies are well characterized in the literature for their unique stability and maintenance of the biological activity even after degradation. In this study, Camelid antibodies showed potent anti-inflammatory effect with reduction of most of the pro-inflammatory mediators. The exact mechanism of this novel anti-inflammatory action of the antibodies needs further investigation, however, it could be postulated that soluble factors in the heat killed *P. acnes* preparation might have a role in acne mediated inflammatory response and a fraction of the generated antibodies from the immunized camels were produced to these inflammatory factors. Compared to the approach of passive immune-protection targeting secreted CAMP factor of *P. acnes* (Liu *et al*., 2011) which revealed potent neutralizing effects of the immunoglobulins against CAMP factor and hence inflammation mediated by acne in mice, we show here an advantage of the antimicrobial and growth inhibitory effects of hyper-immune whey of camels.

Passive immunization by the transfer of antibodies from challenged living organisms do have many advantages over active immunization (Dobano *et al*., 2018). Not only the high specific activity but also unlike active immunization (vaccines), biological effects of passive immunization are immediate and can be of value where symptoms have already occurred. Thus, passive neutralization of anti *P. acnes* antibodies may benefit patients who have already developed acne. The use of hyper-immune whey of camels against acne provide the potential of adjustment of the administered dose based on the severity of disease and it can be easily combined with other therapies. Additionally, unlike active immunization, which requires time to induce protective immunity and depends on the host's ability to mount an immune response, hyper-immune whey of camels can theoretically confer protection regardless of the immune status of the host (Abu-Qatouseh *et al*., 2019).

In conclusion, the constituents of immunized camel milk whey AMPs (i.e. polyclonal anti-*P. acnes* antibodies, peptidoglycan recognition proteins (PGRPs), and lactoferrin) possess significant antimicrobial and anti-inflammatory activity against *P. acnes*. Topical formulae including; facial wash, serum, cream and gel enriched with immunized camel milk whey were developed and their activity against *P. acnes* was evaluated. In conclusion, immunized camel milk whey that has been

developed in this research exerts significant antimicrobial and anti-inflammatory activity against *P. acnes* and the unique composition formulated has been patented.

7.2 Future Work

7.2.1 Conducting Clinical Trials on Human Subjects

To build up on the research work that has been done, and to be able to meet the requirements and regulations for registering the new topical composition containing Camel milk whey enriched with anti-*P. acnes* antibodies as a pharmaceutical over-the-counter formula, a clinical study will be designed to observe, inspect, and document the efficacy of the newly developed formula on human subjects suffering from Acne Vulgaris. Initially, twelve subjects will be enrolled in the trial, and will be organized into four categories based on the stage of acne that they present with; (1) comedonal non-inflammatory acne, (2) mild inflammatory acne, (3) moderate inflammatory acne, (4) severe inflammatory acne. Such categorization is important to establish a correlation between the response obtained and the formula being used, and to determine the stage of acne that exhibits the best response to the formula with the aim to identify the extent to which the formula exerts its efficacy; i.e. efficacy against moderate-to-severe acne or limited efficacy restricted to mild-to-moderate acne.

The plan is to recruit 3 patients with comedonal non-inflammatory acne, 3 patients with mild inflammatory acne, 3 patients with moderate inflammatory acne, and 3 patients with severe inflammatory acne, with equal distribution between males and females, and ages ranging between 19-32; i.e. millennials. All the ethical requirements essential for conducting such a study will be fulfilled, and a dermatologist will be contracted to supervise the work. The subjects will be scheduled to apply the formula two times a day; in the morning and at night.

Clinical response will be assessed every two weeks, and improvement will be judged based on the number of lesions and the relief of inflammation and erythema in existing lesions. To document the results, photographs will be taken at baseline and after 6 to 7 weeks of treatment with the formula.

7.2.2 Formulation of New Cosmeceutical Compositions Enriched with Immunized Camel Milk Whey

Additional formulas based on camel milk whey enriched with anti-*P. acnes* antibodies can be developed the same way that the four cosmeceutical formulas presented in this research were developed. The new formulas will include; facial mask, shower gel, make-up removing wipes, toner, etc. Basically, the aim is to cover the everyday essentials in the maintenance and prevention of Acne Vulgaris.

7.2.3 Using the Results of this Research Work to Develop Formulas Active against other Types of Bacteria

The work done in this research and the results obtained can be extrapolated and further applied to other types of bacteria (apart from *P. acnes*), as the concept and science behind the experiments allows for that. A literature review and market study on Infectious Gastritis and the bacteria *Helicobacter pylori* have been done, and the outcomes are encouraging. The development of a nutraceutical formula based on Camel milk whey enriched with anti-*H. pylori* antibodies is possible following a similar approach to the one proposed in this research work. In brief, female lactating Camels will be immunized with a vaccine containing *H. pylori* antigens, and the whey will be collected and the antibody titer will be assessed to further use the whey to develop a syrup formula and a capsule formula.

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