Biocontrol of Pathogenic Bacteria in Egyptian Cheeses by Bacteriophages

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This Thesis is Dedicated

to

My Family

for Their Endless Love,

Support and Encouragement

Statement of Originality

The contents of this thesis are all my own work, and any quotation from, description of, or the work of others is fully acknowledged by reference to the sources, whether published or unpublished.

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Abstract

Raw milk cheeses such as Karish, Domiati and Ras are among the most consumed cheeses in Egypt; however, they have been a vehicle for transmission of *Escherichia coli* and *Staphylococcus aureus* pathogens. Improving safety without compromising the natural raw milk microbiota and sensorial characteristics requires an alternative to chemical antimicrobials. Bacteriophages are a promising natural tool that can specifically kill bacterial pathogens without affecting the beneficial microbiota; however, far too little attention has been paid to testing phage efficacy against *E. coli* and *S. aureus* strains at more challenging physicochemical conditions and in raw milk cheeses. The main objectives of this study were to evaluate the effect of raw milk cheese physicochemical conditions, non-targeted milk-associated microbiota and cow's milk fat separation on *E. coli* and *S. aureus* phages role as biocontrol agent, in order to inhibit *E. coli* and *S. aureus* growth in Karish, Domiati and Ras cheeses.

Among four *E. coli* and four *S. aureus* tested phages, *E. coli* T4, *S. aureus* EBHT and *S. aureus* K2 phages were selected based on their high lytic ability at 10^5 PFU/ml in liquid medium, host range and high biocontrol activity under Egyptian raw milk cheeses physicochemical conditions: temperature (4, 24, 37 and 45 °C), pH (4.2, 5.1, 5.9 and 6.7) and salt concentration (0.5, 4, 8 and12 %).

E. coli phage T4 and *S. aureus* phage cocktail (1:1 EBHT and K2) were able to reduce the initial host contamination below the detection limit (≤ 10 CFU/ml) after 2 h when they were initially independently tested against 10^4 CFU/ml host strain in sterilized whole and skimmed milk. However, higher phage concentration at 10^8 PFU/ml was required to reach the complete elimination of host in raw milk, due to the effect of present non-target milk components.

Although there were no observed differences in phage lytic ability in sterilized whole and skimmed milk, *E. coli* phage T4 showed higher ability to reduce ($p \leq 0.05$) inoculated and indigenous *E. coli* in raw skimmed milk (3.6 log CFU/ml) compared to raw whole milk (4.6 log CFU/ml) after 24 h. Similar observation was found with *S. aureus* phage cocktail; however, due to their wider host range, inoculated and indigenous *S. aureus* were not detectable after 2 h in raw skimmed milk, while in raw whole milk inhibition was only observed after 24 h.

Finally, *E. coli* phage T4 and *S. aureus* phage cocktail (1:1 EBHT and K2) were applied *in situ* as biocontrol agents in Karish, Domiati and Ras cheeses manufactured from temperature-abused raw skimmed (Karish) and raw whole (Domiati, Ras) cows' milk, with initial microbial populations of 5 to 6 log CFU/ml. *E. coli* phage T4 was able to decrease inoculated and naturally present *E. coli* to ≤ 2 log CFU/g after 15, 1 and 90 days in Karish, Domiati and Ras, respectively. Interestingly, *S. aureus* phage cocktail showed a high ability to reduce *S. aureus* (inoculated and indigenous) and *Staphylococcus* spp. (indigenous) below the detection limit (≤ 10 CFU/g) after 1, 1 and 60 days in Karish, Domiati and Ras, respectively. This research shows that selected phages can be applied as effective biocontrol agents against *E. coli* and *S. aureus* in raw milk cheeses without affecting the cheese fermentation process.

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List of Abbreviations

Abbreviation	Definition
FAO	Food and Agriculture Organization
OECD	Organization for Economic Co-operation and Development
CAGR	Compound Annual Growth Rate
NSLAB	Non-Starter Lactic Acid Bacteria
LAB	Lactic Acid Bacteria
EHEC	Enterohemorrhagic *E. coli*
ETEC	Enterotoxigenic *E. coli*
EPEC	Enteropathogenic *E. coli*
EIEC	Enteroinvasive *E. coli*
EAEC	Enteroaggregative *E. coli*
EMB	Eosin Methylene-Blue
TBX	Tryptone Bile X-Glucuronide
A_w	Water Activity
ISO	International Organization for Standardization
FDA	Food and Drug Administration
CA	Caprylic Acid
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
ICTV	International Committee on Taxonomy of Viruses
ICVCN	International Code of Virus Classification and Nomenclature
RBPs	Receptor Binding Proteins
PG	Peptidoglycan
WTA	Wall Teichoic Acid
LPS	Lipopolysaccharides
OMP	Outer Membrane Proteins

MOI	Multiplicity of Infection
RTE	Ready-to-eat
HTST	High-Temperature-Short-Time
LTLT	Low-Temperature-Long-Time
UHT	Ultra-High-Temperature
Ig	Immunoglobulin
GRAs	Generally Recognized as Safe
RH	Relative Humidity
DSMZ	German Collection of Microorganisms and Cell Cultures
ATCC	American Type Culture Collection
NCTC	National Collection of Type Cultures
NCIMB	National Collection of Industrial Food and Marine Bacteria
MRS	De Man, Rogosa and Sharpe
SM	Saline Magnesium
NA	Nutrient Agar
LB	Luria Bertani
BHI	Brain Heart Infusion
PCR	Polymerase Chain Reaction
STEC	Shiga toxin-producing *E. coli*
LEE	Locus for Enterocyte Effacement
CPS	Coagulase-Positive-Staphylococci
CNS	Coagulase-Negative-Staphylococci
IDF	International Dairy Federation
AOAC	Association of Official Agricultural Chemists
TPA	Texture Profile Analysis
WHO	World Health Organisation
MIC	Minimum Inhibitory Concentration
TBC	Total Bacterial Count

Chapter One: Introduction

1.1 Research Motivation

In Egypt, the consumption of fresh dairy products (un-processed, not plant-based) is expected to increase from 1099 ton (2017 – 2019) to 1359 ton (2029) by average of 2 % annual growth (FAO/OECD, 2020). Moreover, Egyptian artisanal cheese market projected a compound annual growth rate (CAGR) of 18 % during the period 2014 to 2019. The continual growth of consumer preference for raw milk cheeses is mainly due to their stronger flavour and lower price compared with processed cheeses (made from pasteurized milk) (Montel *et al.*, 2014; Yoon, Lee and Choi, 2016) and is also driven by income and population growth (European Union, 2019). Karish, Domiati and Ras are the most popular Egyptian artisanal cheeses; however, their production relies on smallholders in rural areas, with manufacture and storage under poor hygienic conditions (Hammad, Hassan and Shimamoto, 2015; Ibrahim and Awad, 2018), where awareness of food safety principles is assumed to be minimal.

Egyptian raw milk cheeses can, therefore, be a vehicle for various foodborne pathogens including *Escherichia coli* (Ombarak *et al.*, 2016), *Staphylococcus* spp. (El-Baradei, Delacroix-Buchet and Ogier, 2007), *Bacillus* spp. (Sameh, 2016), *Campylobacter jejuni* (El-Sharoud, 2009) and *Salmonella* spp. (Ibrahim, Sharaf and El-khalek, 2015) due to faecal contamination during milking and/or poor hygiene during manufacture (Ombarak *et al.*, 2016). This problem poses a significant threat to both human health and the food industry since those pathogens could cause a serious illness in humans (Bagoury, Shelaby and Saied, 2019). Improving Egyptian raw milk cheeses food safety is, therefore, imperative in order to decrease food-related illnesses or hospitalizations, medical expenses and lost work productivity caused by major foodborne pathogens, and

also to open new markets for Karish, Domiati and Ras exportation. However, the extensive use or misuse of antimicrobial agents has promoted the spread of antibiotic-resistant pathogens (Gómez-Aldapa *et al.*, 2016; Moura *et al.*, 2018).

Although recent studies have focused on using alternatives to antibiotics for controlling foodborne pathogens, such as bacteriocins (Abd-Elmonem *et al.*, 2022), essential oils (Hussien *et al.*, 2019) and protective cultures (Allam *et al.*, 2017), to date there is no adequately designed method to inhibit pathogen growth in raw milk cheese without compromising the natural milk microbiota or cheese sensorial characteristics. Interestingly, bacteriophages offer an extreme specificity to the host strain, which could be a promising alternative to conventional methods for pathogen control (Chang *et al.*, 2019) without affecting either the natural food (Bielmann *et al.*, 2015) or human commensal microbiotas (Carlton *et al.*, 2005).

Since the discovery of phages in the early 20th century, they have been used to control foodborne pathogens for clinical applications (Domingo-Calap and Delgado-Martínez, 2018), ready-to-eat foods (Perera *et al.*, 2015), raw foods (Clavijo *et al.*, 2019) and food manufacturing systems (Gouvêa *et al.*, 2015). Phage application in dairy environment is yet to receive the same interest as other applications, which may be due to the complexity of milk, where the effect of milk components on phage biocontrol activity are not well elucidated.

Therefore, in this study the effect of a wide range of physicochemical conditions and milk components either in whole or skimmed, heat treated or raw milk are initially investigated to assess phages behaviour under those conditions and to select the ideal phages to be applied in Karish, Domiati and Ras cheeses as biocontrol agent.

1.2 Research Objectives & Hypotheses

1.2.1 Hypothesis or research question

Can the use of bacteriophages improve the safety of Egyptian raw milk cheeses without compromising the manufacture process and product quality?

1.2.2 Objectives of the research

The primary motivation of this study was to control foodborne pathogens in Egyptian raw milk cheeses, without compromising the manufacture process and product quality, by using bacteriophages, and to address some of the challenges of phage application in the dairy environment. In doing so, two high level research objectives were established: (1) Characterise selected bacteriophages and determine the ideal protocol for phage application as biocontrol agent in cheese, (2) Examine the limitations and success of bacteriophages for controlling *E. coli* and *S. aureus* strains in the dairy environment.

In order to thoroughly address the two above-mentioned general research objectives, several specific research goals were developed. With respect to objective (1) the following research goals were pursued:

i. Isolate and identify *E. coli* and *S. aureus* strains from raw milk.
ii. Evaluate the effectiveness of bacteriophages against reference and isolated *E. coli* and *S. aureus* strains.
iii. Monitor the physicochemical conditions during manufacture and storage of three different types of Egyptian cheeses (Karish, Domiati and Ras) in small scale laboratory conditions.
iv. Evaluate the performance of phages against *E. coli* and *S. aureus* strains under Egyptian raw milk cheese physicochemical critical conditions.

The specific research goals addressed for objective (2) were as follows:

i. Evaluate *in vitro* long-term stability of selected bacteriophages in dairy environments.
ii. Evaluate the effect of raw milk-associated microorganisms and cow's milk components on *E. coli* and *S. aureus* phage lytic activity.

iii. Determine appropriate bacteriophage application point(s) during small scale cheese manufacture to maintain bacteriophage biocontrol efficacy.

iv. Quantify the *in vivo* efficiency of bacteriophages to control pathogen growth during Egyptian raw milk cheeses manufacture and storage.

1.3 Thesis Structure

Following the introductory Chapter 1, which outlines the research motivation, objectives and hypothesis of the research, a critical literature review is undertaken relevant to the presented study in the thesis (Chapter 2). In particular, previous research on the existence of foodborne pathogens in raw milk cheeses and the relevant methods used for controlling foodborne pathogens are presented, and the limitations of each method are criticised and evaluated. In addition, the applications of bacteriophages as biocontrol agents in food systems are also discussed. Chapter 3 presents a detailed description of the main methods and materials used throughout the entire research, including experimental procedures and analytical techniques.

The results in Chapters 4 to 8 in the thesis are presented in chronological order of completion, which is rational to the building structure necessary for each step of the study. Finally, a general discussion is presented in Chapter 9, which includes the impact of the results of the study and the novel contribution of the research to scientific knowledge, as well as recommendations and future research directions.

Chapter Two: Literature Review

2.1 Introduction

This chapter provides a critical review of the literature related to the presented study. This literature review covers background information on raw milk Egyptian cheeses and related food safety issues, and describes the possible pathogen control methods, and their limitations. Thereafter, bacteriophage structure, recent classifications and life cycle description in relation to food application is discussed. Techniques of phage application in food are also summarised with discussion of their gaps or obstacles, in addition to the possible challenges that could be faced during phage biocontrol applications in dairy environments.

2.2 Artisanal Cheeses

Cheese is considered to be one of the most popular dairy products throughout the world (OECD/FAO, 2018). Due to the implementation of more restricted regulations and legislation for the sale of raw drinking milk and unpasteurised dairy products, most cheese manufacture has evolved from artisan (using raw milk or pasteurized) processes to technological (using pasteurized milk) processes (Kamimura *et al.*, 2019). However, consumer preference for artisanal cheeses is continually growing, due to their unique flavour nuances, variety and lower price (mainly in developing countries), compared to pasteurized milk cheese (Montel *et al.*, 2014; Yoon, Lee and Choi, 2016). Furthermore, in developed countries there is increasing interest in natural products without chemicals or preservatives, from consumers that promote an ‘’organic’’ life style (Dupas *et al.*, 2020). The unique characteristics of artisanal cheeses are governed mainly by the naturally existing microbiota in raw milk and

natural milk enzymes (Franciosi *et al.*, 2011; Westling *et al.*, 2016). Several studies have tried to identify the accompanied species in raw milk microbial community, have found that the most widespread and dominant species in raw milk are *Lactococcus lactis*, *Lactococcus cremoris*, *Streptococcus thermophilus*, *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Enterococus faecalis* and *Enterococus faecium* (Montel *et al.*, 2014; Riquelme *et al.*, 2015; Kamimura *et al.*, 2019). These species, termed non-starter lactic acid bacteria (NSLAB), that are the major source of perceived varied cheese sensory attributes, due to their complex proteolytic or lipolytic action on milk proteins, fat and carbohydrates during fermentation (Ayad *et al.*, 2004; Khattab *et al.*, 2019). However, to date the synergy between species that leads to the diversity in cheese types within different regions is unknown.

2.2.1 Egyptian raw milk cheeses

Interestingly, cheeses made from raw milk are more popular with Egyptian consumers (Allam *et al.*, 2017; Ibrahim and Awad, 2018). Particularly, raw milk is a very important portion of the Egyptian daily diet, as 72 % of raw milk production is directed to Egyptian milk consumption (unprocessed and unpacked form) and raw milk cheese making (European Union, 2019; Ismail, 2021). Karish, Domiati and Ras are the main Egyptian artisanal cheeses, and they are the most popular cheeses not only in Egypt but also in other Arab countries (Ismail *et al.*, 2014; Hammam *et al.*, 2020).

2.2.2 Technological properties of Egyptian artisanal cheeses

2.2.2.1 Karish cheese

Karish is classified as, a fresh, soft (un-ripened) and acid coagulated cheese, broadly similar to Queso fresco, Queso Blanco and Cottage cheese (Fox, Guinee and Cogan, 2000; Ibarra-Sánchez, van Tassell and Miller, 2018).

It is manufactured traditionally from raw cow's milk by setting the milk in a container overnight at room temperature (24 ± 2 °C), during which the fermentation process is achieved by the milk natural microflora. After 24 h the milk fat is separated by removing the top acidic cream layer, leaving lactic

defatted milk, which is called Laban Rayeb (Abou-Donia, 2008). The lactic defatted milk is then transferred to cheese cloth for another 24 h to remove whey, after which the cheese is salted by adding 0.5 – 2 % (of cheese weight) table salt. After cheese cutting, it can be consumed fresh or stored for up to 14 days at refrigerated conditions (Allam *et al.*, 2017). Karish cheese is considered a healthy food product due to its low fat and salt content (Todaro, Adly and Omar, 2013).

2.2.2.2 Domiati cheese

Domiati is a rennet coagulated, soft (internal bacterially-ripened), high salt cheese, close to Feta and Telemes (Greece) (Fox, Guinee and Cogan, 2000). Domiati is made from raw whole cows' or buffalos' milk or a mixture of both, and is stored in salted whey or brine solution. Domiati can be consumed fresh or usually after one to three months of ripening (Ayad, 2009). Domiati cheese differs from any other type of cheese, as It is the only cheese where salt is added to milk directly before fermentation or coagulation, rather than on the cheese itself (Guinee and Sutherland, 2022).

The manufacture process can be summarized as follows: a) warming raw milk to 37 °C followed by addition of salt by 5 to 15 % (from milk weight); b) the required amount of rennet enzyme to complete coagulation in 3 h is calculated (according to manufacturer); however, due to the presence of high salt content in milk, the calculated amount of rennet is doubled; c) the formed curd is then transferred to wooden box with cheese cloth to separate whey; d) the resultant curd is left overnight with gentle press for further whey separation; e) the cheese is cut into cubes and stored in pasteurized salted whey or brine solution for one to three months at 4 °C (Soryal *et al.*, 2004).

2.2.2.3 Ras cheese

Ras is a rennet coagulated, internal bacterially-ripened hard cheese. It is manufactured from raw milk or a mixture of raw and pasteurized milk, which is considered as the most suitable way to control the NSLAB population and acid development during cheese processing (Ayad *et al.*, 2004). It originated from and is close to the Greek cheese variety "Kefalotyri" (Hofi *et al.*, 1970; Hammam *et*

al., 2020). It is sold locally in markets under the name of Roomy or Torky (Ismail *et al.*, 2014).

The manufacture process can be summarized as follows: a) raw milk is warmed to 32 °C and the annatto pigment is added to give the cheese its yellow colour; b) the milk is then left for 30 min for natural microflora maturation, after which enzymatic rennet coagulation is performed for 30 to 45 min followed by curd cutting to cubes (1 cm^2); c) the cubes are then scalded by increasing temperature gradually to 42 °C with stirring; d) after around 50 min the curd is transferred to a round moulds and pressed overnight; e) Ras cheese is stored in ripening rooms for at least three to six months for ripening and flavour development (Hofi *et al.*, 1970; Awad, 2006).

2.2.3 Physicochemical properties of Egyptian artisanal cheeses

As previously discussed, Karish, Domiati and Ras are representative of diverse cheese families with varied technological characteristics, and subsequently a wide range of physicochemical properties. Moreover, understanding the accompanied physicochemical conditions throughout food production and subsequent storage is crucial for developing product chemical (El Soda and Awad, 2011), microbiological (Ibrahim and Awad, 2018) or technological (Youssef *et al.*, 2019) quality.

The Karish, Domiati and Ras physicochemical properties during storage are summarised in Table 2.1. Karish has the lowest pH value (4.6) in comparison with Domiati (6.4) and Ras (5.9) at first day of storage. Furthermore, these pH values decrease gradually until the end of storage. On the other hand, Domiati is recognized by the high salt content (3.0 – 8.0 %) between most cheese types (El-Baradei, Delacroix-Buchet and Ogier, 2007) and Karish has the lowest salt content (0.2 - 1.5 %). Additionally, Ras has the lowest moisture content (23.0 – 33.0 %), while Karish has the highest moisture content (64.8 - 71.7 %). The moisture content of Karish, Domiati and Ras cheeses decreases gradually with increasing protein and fat percentages during storage time. Notably, little attention has been paid to studying the effect of changes in milk and cheese chemical composition on water activity, that could modulate the growth of bacteria in cheese (Coffey *et al.*, 2011; Al-Nabulsi *et al.*, 2020).

Table 2.1. Egyptian artisanal cheeses physicochemical properties range during storage time.

Cheese	Storage Time*	pH	Protein %	Fat %	Moisture %	Salt %	References
Karish	1 to 15 days	4.3 – 4.6	17.5 – 22.5	2.2 – 5.8	64.8 – 71.7	0.2 – 1.5	(Sameh, 2016) (Allam *et al.*, 2017)
Domiati	3 to 6 months	5.2 – 6.4	14.0 – 15.6	18.0 – 23.0	53.0 – 62.0	3.0 – 8.0	(Ayad, 2009) (Ibrahim and Awad, 2018)
Ras	3 to 12 months	5.2 – 5.9	22.0 – 26.0	28.0 – 30.0	23.0 – 33.0	1.7 – 4.0	(Hofi *et al.*, 1970) (Awad, 2006)

*Time at which the cheese is ready for consumption up to the end of shelf life.

2.2.4 Food safety challenges of Egyptian cheeses made from raw milk

Food safety is one of the key areas of focus for international food marketing, because it has significant impacts in public health (Gizaw, 2019). Milk is a highly valuable and nutritional food, where, the milk quality is determined by several aspects such as: chemical composition and microbial hygiene. Due to the complexity of milk chemical composition and high-water activity, milk considered as an excellent medium for the growth of several microorganisms such lactic acid bacteria and zoonotic pathogens (Ibrahim, Sharaf and El-khalek, 2015; Andrew and Fera, 2016). These microbial pathogens get access to milk either by direct secretion from udders with clinical or subclinical mastitis or by contamination from the environment during handling of raw milk or faecal contamination (Rosengren *et al.*, 2010; Ombarak *et al*., 2016; Rios *et al.*, 2019).

As Karish, Domiati and Ras cheeses are mainly manufactured by rural families and sold from door-to-door or sold in local vendors (Ombarak *et al.*, 2016) under uncontrolled hygienic conditions (European Union, 2019), where workers do not handle food hygienically and make them more susceptible to microbial hazards. Consequently, appropriate handling of dairy products is essential to effectively avoid contamination (Ahmed, Shimamoto and Shimamoto, 2014). However, food safety information in Egypt and most African countries is insufficient related to limited documentations and information traceability (Paudyal *et al.*, 2017; Kamal *et al.*, 2018). In this respect, the microbiological hazards of raw milk and associated products has been detected worldwide (Verraes *et al.*, 2015; de Medeiros Carvalho *et al.*, 2019; Mollayusefian *et al.*, 2021).

The Egyptian Organization for Standardization and Quality Control currently recommend cheese making using pasteurized milk to improve the manufacture process and cheese food safety (Mahrous, 2014). However, due to the high consumer preference for raw milk cheeses, to date a high proportion of the cheeses produced in Egypt are still manufactured from raw milk in rural areas. That is also linked to the recent report by European Union (2019) who observed the unharmonised food safety legislation during investigation of Egyptian food and beverage market. To date there are no available specific

starter cultures to produce Karish, Domiati or Ras cheeses; and producer still need to produce those cheeses from raw milk.

To facilitate the production of these artisanal cheeses on a larger scale from pasteurized milk, a few researchers have attempted to isolate the exact strains responsible for the main flavour in each raw milk cheese. For example, Allam *et al.* (2017) demonstrated that using two *Lactococcus* spp. (*L. lactis* subsp. *cremoris* kM746 and *L. lactis* KM721) isolated from traditional Karish cheese could successfully produce Karish cheese made from pasteurized milk with physicochemical properties similar to the traditional one. However, the sensory evaluation results showed higher smell, taste and appearance grades for raw milk produced cheese compared to the treated cheese; in addition, panellists were able to recognise the raw milk produced cheese from other treatments.

On the other hand, Ayad *et al.* (2004) are the only researchers that have tried to define the flavour compounds of Ras cheese, identifying 68 volatile compounds, including alcohols, aldehydes and ketones. In 2006 and 2007, the same research team identified 18 different cultures including *Lactobacillus delbrueckii* subsp. *lactis* 119TH, *Lactobacillus acidophilus* 83ST, *Lactobacillus paracasei* subsp. *paracasei* 27ST, *Lactobacillus helveticus* 120B, and *Enterococcus faecium* 102E, 136E and 241E (isolated from Egyptian dairy products), and applied these as starter cultures in Ras cheese manufacture (Awad, 2006; Awad, Ahmed and Soda, 2007). Although the obtained sensory evaluation of the Ras cheese showed that the starter mix got the highest grade, the panellists stated that they preferred the taste of the artisanal Ras cheese.

2.3 Prevalence of Foodborne Pathogens in Egyptian Artisanal Cheeses

Although, raw milk and artisanal cheeses are a source of beneficial lactic acid bacteria (LAB), they can also be a public health risk when contaminated by foodborne pathogens (Claeys *et al.*, 2013). A number of food safety outbreaks in raw milk dairy products have been recently reported in different worldwide regions including: USA (Haymaker *et al.*, 2019), Italy (Bianchi *et al.*, 2013), Germany (Böhnlein *et al.*, 2021), Mexico (Canizalez-Roman *et al.*, 2019), Algeria (Titouche *et al.*, 2019), Brazil (Aidar-Ugrinovich *et al.*, 2007), Bangladesh (Jahan *et al.*, 2015), Ireland (Lourenco *et al.*, 2020) and England (Willis *et al.*, 2022).

In particular, the recent evidence showed that the most reported foodborne pathogens are *Escherichia coli* (Dell'Orco *et al.*, 2019), *Staphylococcus aureus* (Alves *et al.*, 2018), *Salmonella* spp. (Bianchi *et al.*, 2013), *Listeria monocytogenes* (Willis *et al.*, 2018) and *Campylobacter* spp. (Kenyon *et al.*, 2020). However, the incidence and level of each pathogen is linked to the cheese type and regional environmental conditions. For example, *Listeria* spp. have been more frequently reported and studied in regions with cold weather conditions, while *E. coli* and *S. aureus* are more reported in regions with hot/dry weather conditions (Shrihari and Negi, 2011; Feliciano *et al*., 2021).

A limited amount of literature has been published on the incidence of foodborne pathogens in Egyptian artisanal cheeses. Table 2.2 shows that *E. coli* and *S. aureus* strains were more prevalent in Egyptian artisanal cheeses. However, several limiting data gaps were noticed such as: non-uniform protocols for sampling, detection of pathogen contamination levels, enterotoxin production and source of contamination, that could contribute to data heterogeneity. Of note, the link between hospitalization and food contamination in Egypt and the African regions is limited and insufficient (Paudyal *et al.*, 2017).

Table 2.2. Reported prevalence of foodborne pathogens in Egyptian artisanal cheeses.

Pathogen	Cheese Type	Number of samples	Prevalence rate %	Contamination Level (log CFU/g)	References
E. coli	Karish	30	33	5.8	(Ibrahim, Sharaf and El-khalek, 2015)
		15	81	3.4 – 5.2	(Sameh, 2016)
		55	74	N.I.*	(Ombarak *et al.*, 2016)
		75	16	N.I.	(Hussien *et al.*, 2019)
	Domiati	50	20	3.6 – 3.8	(Ibrahim, Sharaf and El-khalek, 2015)
	Ras	60	21.7	N.I.	(Ombarak *et al.*, 2016)
		75	5.3	N.I.	(Hussien *et al.*, 2019)
S. aureus	Karish	30	93	4.8 – 5.7	(Kamal, Bayoumi and Abd El Aal, 2013)
		30	66	5.5	(Ibrahim, Sharaf and El-khalek, 2015)
		30	80	4.3 – 5.0	(ElLeboudy, Amer and Shahin, 2015)
		15	10	1.8 – 2.7	(Sameh, 2016)
		30	8	N.I.	(Dawoud *et al.*, 2018)
		50	38	4.0 – 4.3	(Abdel-Hameid Ahmed *et al.*, 2019)
	Domiati	40	N.I.	7.0 – 7.5	(El-Baradei, Delacroix-Buchet and Ogier, 2007)
		50	60	4.4	(Ibrahim, Sharaf and El-khalek, 2015)
		50	14	2.1 – 2.3	(Abdel-Hameid Ahmed *et al.*, 2019)
	Ras	30	80	5.2 – 6.0	(ElLeboudy, Amer and Shahin, 2015)
		50	48	4.0 – 5.6	(Abdel-Hameid Ahmed *et al.*, 2019)
Bacillus spp.	Karish	15	27	4.2 – 5.6	(Sameh, 2016)
Salmonella spp.	Karish	120	0.4	N.I.	(Ahmed and Shimamoto, 2014)
		30	6.7	N.I.	(Ibrahim, Sharaf and El-khalek, 2015)
	Domiati	120	0.13	N.I.	(Ahmed and Shimamoto, 2014)

*N.I.: Not identified in the presented study.

In addition, Bagoury, Shelaby and Saied (2019) found that 35.6, 14.2 and 14.2 % of isolated *E. coli* strains were characterized as enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E.coli* (ETEC) and enteropathogenic *E. coli* (EPEC), respectively. In this approach, Hussien *et al.* (2019) found that 11 (38 %) *E. coli* isolates from Karish and Ras were positive for virulence genes *stx1* and *stx2*. Furthermore, Ombarak *et al.* (2016) reported that isolated *E. coli* strains carrying virulence genes were recorded in 34 (47.2 %) raw milk, 26 (47.3 %) Karish cheese and 9 (15 %) Ras cheese samples.

Although the growth of pathogenic bacteria during manufacture and storage can be decreased or suppressed by the growth of LAB, development of acidity, salt concentration and moisture content (Callon, Arliguie and Montel, 2016; Allam *et al.*, 2017), many researchers have reported that *E. coli* and *S. aureus* strains could survive under various cheese manufacture and storage conditions (Maher *et al.*, 2001; Peng, *et al.*, 2013a; Ibrahim *et al.*, 2020; Al-Nabulsi *et al.*, 2020). Therefore, the development and evaluation of efficient strategies to control foodborne pathogens in artisanal cheeses without modifying cheese characteristics is a necessity.

2.3.1 *Escherichia coli*

E. coli is a short rod-shaped, Gram-negative, non-spore forming bacterium belonging to the Enterobacteriaceae family. Optimum growth is between pH 6 and 7.5, and it is able to grow over a wide pH range between 4.5 and 9 (Callon, Arliguie and Montel, 2016). *E. coli* has been recovered easily from different milk samples using generic or selective media incubated at 37 °C. MacConkey or Eosin Methylene-Blue (EMB) agar are the typical generic media used to recover *E. coli* from different dairy samples (Guzman-Hernandez *et al.*, 2016; Miliotis, 2018; Ngaywa *et al.*, 2019). *E. coli* colony shape is circular and smooth with an entire edge when grown on agar media. A rapid method using selective chromogenic media such as Tryptone Bile X-Glucuronide (TBX) has been used successfully to differentiate *E. coli* from coliforms (Verhaegen *et al.*, 2016; Willis *et al.*, 2022).

Pathogenic *E. coli* can be categorized into one of five main groups according to their mechanism of virulence: enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enterohemorrhagic (EHEC), and enteroaggregative (EAEC) (Miliotis, 2018). A considerable amount of literature has showed that EPEC is the most detected serogroup in a wide variety of raw milk and dairy products (Beraldo *et al.*, 2014; Álvarez-Suárez *et al.*, 2016; Ribeiro *et al.*, 2019; Rios *et al.*, 2019). The EPEC adherence to the epithelial cells is related to a 94 kDa outer membrane protein called intimin. The *eae* gene that encodes intimin was first reported by Jerse and Kaper (1991). This gene is present in all EPEC and EHEC strains; moreover, several studies have demonstrated that the *eae* gene is an important virulence factor for EPEC molecular identification (Aidar-Ugrinovich *et al.*, 2007; Wani *et al.*, 2009; Haymaker *et al.*, 2019).

2.3.2 *Staphylococcus aureus*

S. aureus is a spherical-shaped Gram-positive bacterium (coccus) that is recognised microscopically in pairs, short chains, or bunched, grape-like clusters. Although *S. aureus* is a mesophile (35 °C optimum temperature), some strains can grow as low as 6 – 7 °C. Its pH growth range is between 4.5 and 9.3, with optimum of 7.0 to 7.5. Staphylococci are unique in their ability to grow at lower water activity (A_w) levels (0.83) than other non-halophilic bacteria. Notably, most *S. aureus* strains are highly tolerant to the presence of salts and can grow over an A_w range between 0.83 and ≥ 0.99, where this ability depends on other growth conditions (Medveov and Valk, 2012; Miliotis, 2018).

S. aureus bacteria can be recovered and differentiated from other staphylococci by their ability to reduce potassium tellurite producing black colonies, coagulase activity, and ability to hydrolyse egg yolk (Medveov and Valk, 2012). On the other hand, enrichment isolation and direct plating are the most commonly used techniques for detection and enumeration of *S. aureus* in dairy samples, where the spread plate technique is recommended due to the higher ability to characterise surface colony appearance than the subsurface colonies encountered with pour plate (ISO, 1999; Mhone, Matope and Saidi, 2011). Furthermore, some strains can produce a highly heat-stable protein toxin

(enterotoxin), that can cause illness in humans (Hennekinne, De Buyser and Dragacci, 2012).

2.4 Control of Foodborne Pathogens

The previously reported incidence of foodborne pathogens in raw milk cheeses highlights the need to find a method suitable for control of pathogen growth throughout manufacture and storage without compromising the natural milk microbiota. Numerous studies have attempted to inhibit the growth of targeted pathogens in cheese by using chemical and natural methods, however application of chemical preservatives such as propionates and sorbates (FDA, 2020) has a negative effect on the raw milk-associated microbiota (Farrokh *et al.*, 2013).

In particular, recent concerns have arisen about emergence of food-borne antimicrobial resistant (AMR) pathogens, due to the overuse of critical human antibiotics to promote animal growth or as animal therapy (Choffnes *et al*., 2012; Godziszewska *et al*., 2018; Nikoloudaki *et al*., 2021). Several studies have identified a significant association between animal/human antibiotics consumption and AMR in food and human (Choffnes *et al*., 2012; Woolhouse *et al*., 2015; Ma *et al*., 2021; Allel *et al*., 2023). Interestingly, Lee (2003) reported that the random amplified polymorphic DNA (RAPD) patterns of six *S. aureus* (MRSA) cow's milk isolates were similar to *S. aureus* (MRSA) human clinical isolates.

In addition, organic farms are not allowed to use antibiotics and efficacious alternative means are limited (D'Amico and Donnelly, 2010). Moreover, several studies investigated the use of natural biocontrol methods such as bacteriocins (Bockelmann *et al.*, 2017; Lourenço *et al.*, 2017) essential oils (Hussien *et al.*, 2019; Dupas *et al.*, 2020) and protective cultures (Aspri *et al.*, 2017; Nikodinoska *et al.*, 2019; Bragason *et al.*, 2020); however, inhibition of foodborne pathogens was difficult to achieve.

Although several biocontrol studies have achieved favourably reduction or complete inhibition of target pathogen in treated cheeses, many unfavourable effects have also been observed depending on the applied method. For example, bacteriocins are not highly specific, so they would affect the growth of many other

Gram-positive or Gram-negative bacteria (according to bacteriocin type), including the technologically important bacterial species (Bockelmann *et al.*, 2017). Lourenço *et al.* (2017) added that application of caprylic acid (CA), nisin (2.5 %) or a mixture of sodium lactate and sodium diacetate individually in Queso fresco cheese led to only 1 log CFU/g reduction of *L. monocytogenes*; besides the consumer panel (n = 67) preferred the control than treated cheese. Likewise, essential oils application in cheese manufacture would add a significant flavour to final product (Dupas *et al.*, 2020).

On the other hand, several researchers have focused on using different bacterial species, mainly LAB strains, to control growth of pathogens such as Shiga toxin-producing *E. coli* (Callon, Arliguie and Montel, 2016), *L. monocytogenes* (Coelho *et al.*, 2014) and *Salmonella* spp. (Bragason *et al.*, 2020). This observed interest could be related to the following: this method usually does not significantly affect the sensory attributes of treated cheese, the contribution between natural or inoculated starter in fermentation process could be achieved. However, a number of limitations of this application are identified and can be summarised as follows: a) the complete elimination of target strain was not achieved at high initial concentration (10^4 to 10^6 CFU/ml) despite the concentration level of protective culture; b) the control activity of applied cultures took 24 h to several days to achieve pathogen inhibition; c) there was observed competition between protective culture and NSLAB. According to previous observations, the potential success for eliminating pathogen at high concentration and suppressing *S. aureus* enterotoxins production is questionable.

Therefore, a more targeted method such as bacteriophages (phages) has been shown to be a promising alternative tool for controlling targeted pathogens (Chang *et al.*, 2019), primarily due to their high specificity without affecting either the natural food (Bielmann *et al.*, 2015) or human commensal microbiota (Carlton *et al.*, 2005).

2.5 Bacteriophage Structure, Classification and Life Cycles

2.5.1 Bacteriophage History

Bacteriophages (phages) are parasites that infect prokaryotes. In 1896, Ernst Hankin was the first scientist to observe the bactericidal properties against *Vibrio cholera* of water filtrated from the Ganges and Jumna rivers in India; however, he concluded that the antiseptic effect was due to the presence of a volatile chemical substance. It was not until 1915 and 1917 that a significant shift occurred through Frederick Twort and Felix d'Herelle, who independently discovered a microbe which invades bacteria and causes their lysis in liquid culture and forms separate patches, which they called plaques, on the surface of the bacteria-inoculated agar (Burrell, Howard and Murphy, 2016). D'Herelle assumed that these invisible microbes were "ultraviruses" that invaded bacteria and multiplied indefinitely in the presence of living cells, and so he called them bacteriophage (from the Greek *phagin*, meaning to devour or eat) (Summers, 2001). However, his conception of phages as viruses of bacteria was not widely accepted until the early 1940s (Kutter and Sulakvelidze, 2004).

2.5.2 Bacteriophage description

The phage particle (virion) consists of a nucleic acid genome (DNA or RNA) enclosed within a protein coat (lipoprotein or capsid; 30 – 160 nm), which combine to form the nucleocapsid. The tail in tailed phages is consist of the tail tube (153 nm) surrounded by a helical sheath both tube and sheath are attached to the base plate at the end of the head (Figure 2.1).

The virions are approximately half double-stranded nucleic acid and half protein by mass, with icosahedral heads assembled from many copies of a specific protein or two. In particular, tailed phages are defined by their very distinct tail morphologies: i) the *Myoviridae* (myo: long contractile), with double-layered contractile tails consisting of a sheath and central tube (Figure 2.1a); (ii) the *Siphoviridae* (sipho: long non-contractile), with long flexible non-contractile tails (Figure 2.1b), (iii) the *Podoviridae* (podo: short tails), with short non-contractile tails (Figure 2.1c), iv) *Ackermannviridae*, with non-enveloped head-tail, a neck without a collar, and a small base plate (Figure 2.1d) (Chibani *et al.*, 2019; Barylski *et al.*, 2020).

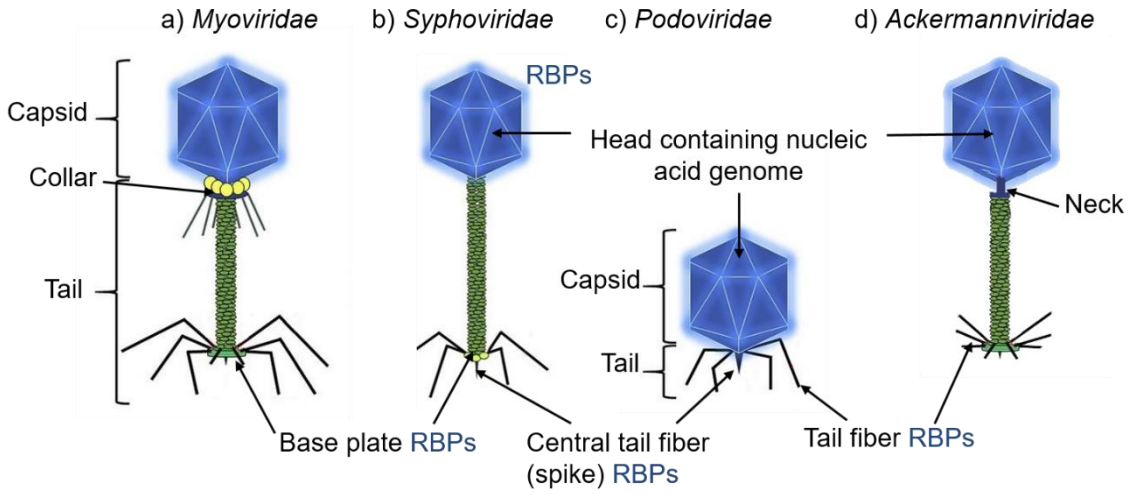

Figure 2.1. Structure of (a) *Myoviruses*, (b) *Sypoviruses*, (c) *Podoviruses* and (d) *Ackermannviridae* phages with identifying the receptor binding proteins (RBPs) (Adapted from: (GarcíaS-Anaya *et al.*, 2020)).

2.5.3 Bacteriophage classification

Since all phages share similar description as stated above, virologists have developed a unified system of classification headed by the International Committee on Taxonomy of Viruses (ICTV) that covers all viruses. The International Code of Virus Classification and Nomenclature (ICVCN) is currently responsible for the nomenclature of viruses and their subclassification, instead of ICTV (Jens, Sheli and Sina, 2014; Walker *et al.*, 2021). Bacteriophages can be classified initially on the basis of: a) type of nucleic acid (DNA or RNA); b) virion size, morphology and structure; c) strategy of viral replication (life cycle) and d) host specificity (Burrell, Howard and Murphy, 2016).

The most recent update (July 2021) of the taxonomic classifications of bacteriophages maintained by the ICTV revealed that bacteriophages are classified into 65 orders, 8 suborders, 233 families, 168 subfamilies, 2606 genera, 84 subgenera, and 10434 species (ICTV, 2021).

In 2020 and 2021, the phage classification was drastically changed mainly the tailed phages classification. A new uniform rule for virus species naming was made, as, the binomial ‘genus-species’ format was made with or without

Latinized species description. According to the latest updates 42 class, 69 order, 318 family, 2440 genus and 12150 species were added to the ICTV classification, however part of this number came from moved or renamed ranking (Walker *et al.*, 2020; Walker *et al.*, 2021). For example, in 2018, *Escherichia* virus T4 was classified under Caudovirales order and *Myoviridae* family; however, in 2021, the species was renamed to *Tequatrovirus* T4 and classified as follow: Class: *Caudoviricetes*; Family: *Straboviridae*; Subfamily: *Tevenvirinae* and Genus: *Tequatrovirus*.

Most of the currently classified bacteriophages are assigned to tailed phages of the realm: *Duplodnaviria*, kingdom: *Heunggongvirae*, phylum: *Uroviricota* and class: *Caudoviricetes*, which contains the majority of widespread foodborne viruses (Maniloff, Ackermann and Jarvis, 1999). Tailed phages were commonly divided into three main families namely *Myoviridae*, *Podoviridae* and *Siphoviridae* (Simmonds and Aiewsakun, 2018), however in 2021 these families are renamed and reclassified as illustrated in Table 2.3.

Table 2.3. Examples of the recent classifications update of some known phages in dairy application.

Species	Renamed to	Family	Subfamily	Genus
Escherichia virus T4	*Tequatrovirus* T4	*Straboviridae*	*Tevenvirinae*	*Tequatrovirus*
Escherichia virus K1E	*Vectrevirus* K1E	*Autographiviridae*	*Molineuxvirinae*	*Vectrevirus*
Escherichia virus T7	*Teseptimavirus* T7	*Autographiviridae*	*Studiervirinae*	*Teseptimavirus*
Staphylococcus virus K	*Kayvirus* kay	*Herelleviridae*	*Twortvirinae*	*Kayvirus*
Staphylococcus virus SA11	*Silviavirus* SA11	*Herelleviridae*	*Twortvirinae*	*Silviavirus*
Staphylococcus virus SA12	*Kayvirus* SA12	*Herelleviridae*	*Twortvirinae*	*Kayvirus*
Bacillus virus Bcp1	*Caeruleovirus* Bcp1	*Herelleviridae*	*Bastillevirinae*	*Caeruleovirus*
Bacillus virus phi29	*Salasvirus* phi29	*Salasmaviridae*	*Picovirinae*	*Salasvirus*
Listeria virus P100	*Pecentumvirus* P100	*Herelleviridae*	*Jasinkavirinae*	*Pecentumvirus*
Listeria virus A511	*Pecentumvirus* A511	*Herelleviridae*	*Jasinkavirinae*	*Pecentumvirus*
Salmonella virus SJ2	*Kuttervirus* SJ2	*Ackermannviridae*	*Cvivirinae*	*Kuttervirus*

(Source: https://ictv.global/taxonomy; accessed on 7 August 2022).

2.5.4 Bacteriophage life cycles

Bacteriophages are intracellular sub-microscopic parasites that are exclusively able to infect bacterial cells, replicate and subsequently suppress their propagation. Phages can also be divided into two main classes (lytic or lysogenic) based on infection pathway.

The phage lytic phase causes cell lysis, whereas the phage becomes integrated into the host genome as a prophage during the lysogenic phase (Kutter and Sulakvelidze, 2004). Interestingly, virulent phages can only multiply and inhibit the host by the lytic cycle. Virulent lytic phages are ideal for phage application as a biocontrol agent due to bacterial cell death during phage multiplication and release of progeny phage (Guenther *et al.*, 2009). In contrast, lysogenic phages (called pro-phage) would make a much better vector in transferring genetic material in recDNA compared to lytic phages. Thus, lysogenic phages could contribute to the spread of unfavorable features by transferring antimicrobial resistance or virulence genes from one cell to another (García *et al.*, 2007; Tabla *et al.*, 2022).

The five phases of lytic cycle (Figure 2.2) could be described as follows: 1) Adsorption of phage to the bacterium (initial contact), in which the bacteriophage irreversibly attaches to a specific receptor site using tail fibers or spike. In addition, this interaction could be mediated according to the amount of phage receptor binding proteins (RBPs), that differ based on phage morphology (Figure 2.1). These receptors are one of the main key factors that determine the specific phage binding (Dowah and Clokie, 2018). The full mechanism is different in Gram-positive and Gram-negative bacteria, regarding the difference in bacterial cell wall structure. Gram-positive bacteria are recognized by the presence of peptidoglycan (PG) anchored wall teichoic acid (WTA) in cell wall and lipoteichoic acid (LTA) (Figure 2.3a), one of the main molecules embedded in complex thick cell wall and interacted with phage RBPs (Xia *et al.*, 2011; Bielmann *et al.*, 2015). However, in Gram-negative bacteria (Figure 2.3b) a variety of associated cell wall structures -mainly lipopolysaccharides (LPS), outer membrane proteins (OMP), pili, flagella and oligosaccharides - are interacted with phage RBPs (Kutter and Sulakvelidze, 2004; Alzamora *et al.*, 2011). Notably, the rate of phage and host bacteria interaction is varied under different growth conditions,

due to the observed changes in autoaggregation and sedimentation of membrane proteins (Scanlan, Bischofberger and Hall, 2017; Dowah and Clokie, 2018).

Of note, several studies have focused on studying the binding interactions between phage and bacteria in *in vivo* systems, however, the interaction mechanism between phage and host in different food matrices remains mostly uninvestigated (Moldovan, Chapman-McQuiston and Wu, 2007; Xia *et al*., 2011; García-Anaya *et al.*, 2020).

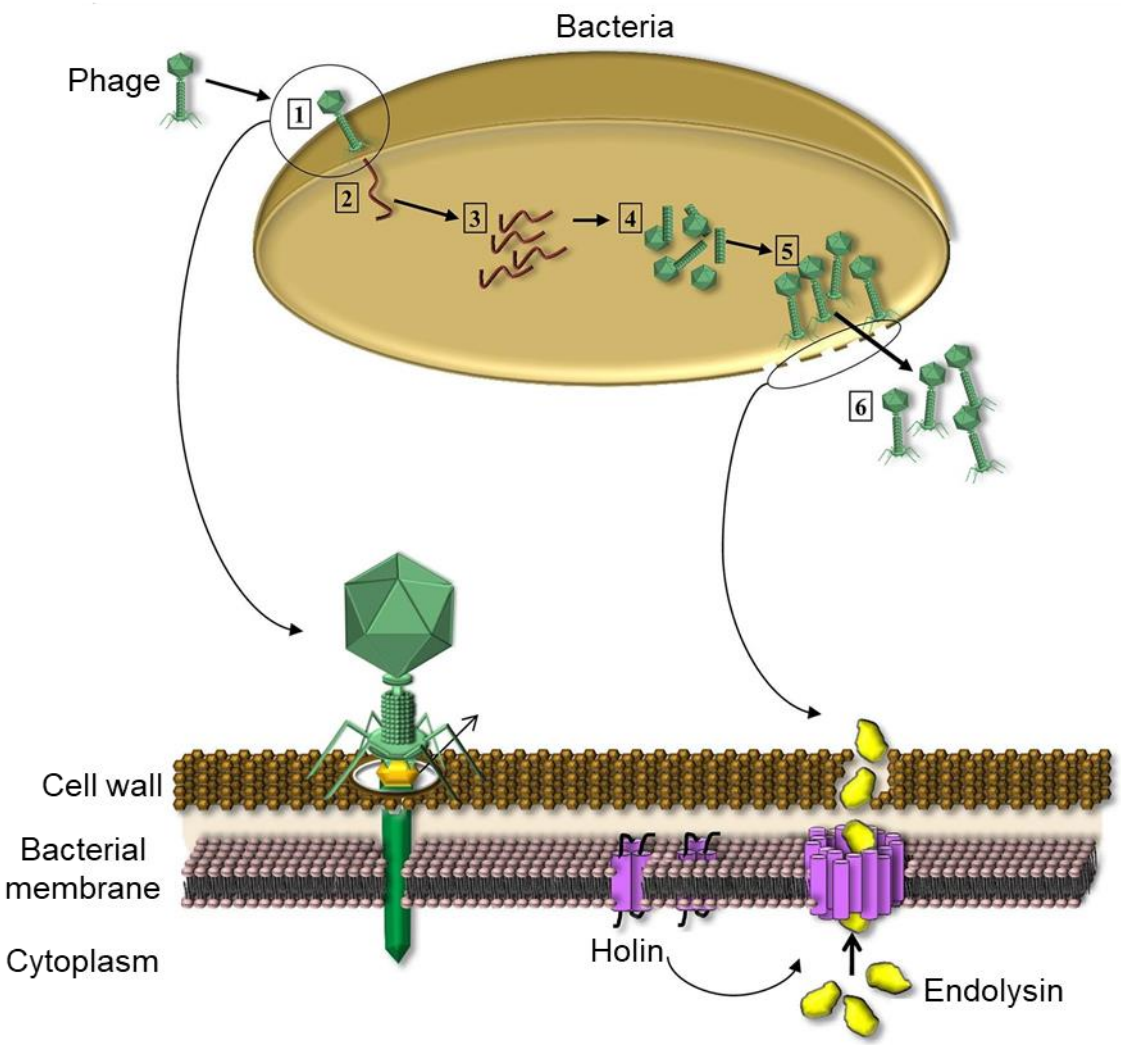

Figure 2.2. Bacteriophage lytic cycle. 1, Phage adsorption; 2, Injection of genetic material; 3, synthesis of phage DNA; 4, maturation; 5, bacterial lysis; 6, phage release. The structure shown is a gram positive bacterium (Adapted from: (Gutiérrez *et al.*, 2018)).

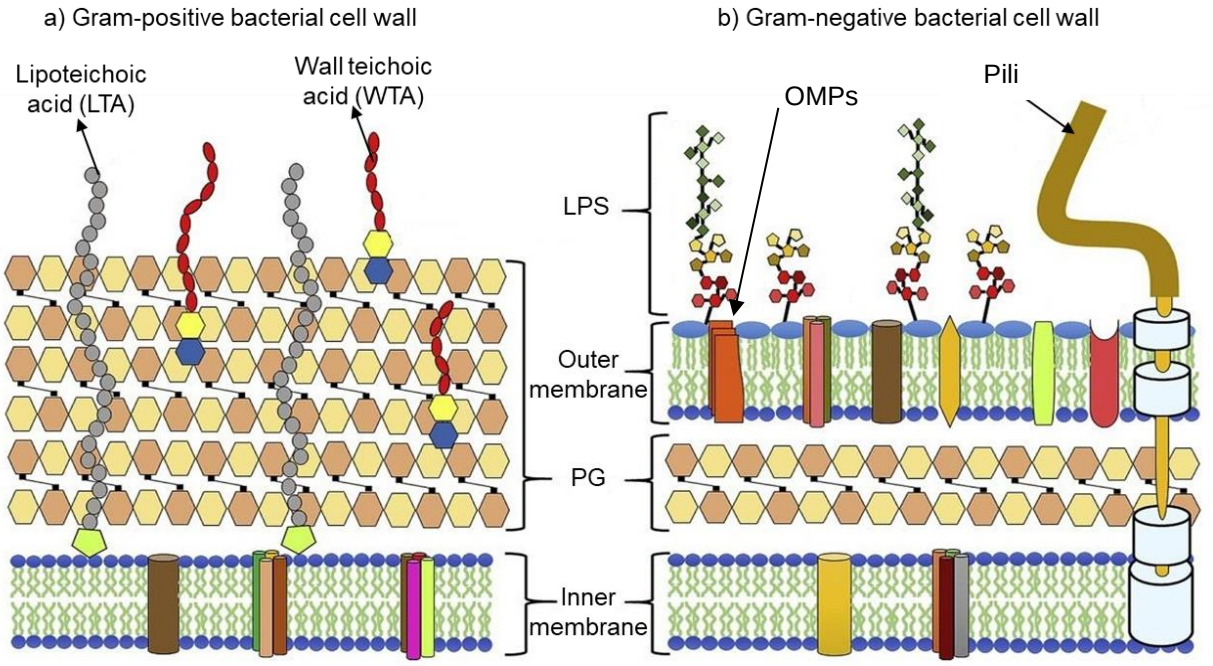

Figure 2.3. Basic elements of Gram-positive (a) and Gram-negative (b) cell wall structure and physiology that are relevant to phage infection process (Adapted from: (García-Anaya *et al.*, 2020)).

The second phase in the phage lytic cycle is (2) injection or penetration of genetic material into the cytoplasm by forming a hollow tube that penetrates into the bacteria, allowing the passage of DNA into the bacterial cell; after that (3) synthesis of phage DNA and alteration of the bacteria metabolic activity; then (4) maturation and assembly of bacteriophage components (DNA, head and tail) to form virions. Finally, bacterial lysis (5) of the host cell and release (6) of the progeny phages, which is accomplished by two proteins: holin (a hydrophobic polypeptide) and endolysin (Figure 2.2). Endolysins and holins are produced at the end of the life cycle. Holins form a pore in the bacterial membrane, allowing the endolysin to reach the peptidoglycan to initiate bacterial lysis (Gutiérrez *et al.*, 2018). The time interval between the entry of bacteriophage and appearance of the first progeny phage (as depicted in Figure 2.2) is called the eclipse phase. The number of phage particles released varies from a few to 500 per cycle,

which depends upon bacteriophage strain, bacterial strain and its metabolic state.

On the other hand, the lysogenic cycle is the ability of the phage nucleic acid to integrate into the bacterial chromosome or other genetic elements without bacterial death. Pseudo-lysogeny occurs when the phage nucleic acid is unstable within the host and the host is subjected to unfavorable conditions such as nutrient limitations and antibiotic presence (Hudson *et al*., 2005); the lytic or lysogenic cycle may then occur when conditions become more favorable (James *et al.*, 2001).

2.6 Bacteriophage Applications in Food Safety

The uses of phage as biocontrol agent against bacterial pathogens are attributed to a number of desired properties, include the following: a) phages are highly specific to species, and they are able to eliminate only their live host bacteria without affecting the remaining microbiota (in contrast to antibiotics) (Hu, Meng and Liu, 2016); b) phage population is self-replicating in presence of host species; c) they are effective even in low doses (Kasman *et al.*, 2002); d) they are regarded as a commensal microorganism in the gastrointestinal tract (Samtlebe *et al.*, 2016); e) they can be isolated from accessible sources including municipal wastewater (El-Dougdoug *et al.*, 2019), soil (Bai, Jeon and Ryu, 2019), dairy plant sewage (Han *et al.*, 2013) and human sewage (Hu, Meng and Liu, 2016).

Several researchers have succeeded in isolating phages from different food ecosystems such as: oyster (Zhang *et al.*, 2018), chicken (Firlieyanti, Connerton and Connerton, 2016; Thung *et al.*, 2019), milk (Kopčáková *et al.*, 2018; Komora *et al.*, 2020), fermented food (Bandara *et al.*, 2012), meat (Thung *et al.*, 2017) and dairy products (Fister *et al.*, 2016b). According to the described phage characteristics, phages have the ability to survive for a long time, propagate rapidly, and have the dynamic balance to remain viable in varied natural ecosystems. The phage high specificity to host bacteria within the same species or across related species, make them an ideal weapon against food-borne pathogens without affecting LAB strains that could be necessary for food processing.

As phages and their host could be normally present in food ecosystem and, thence be a part of natural microbiota; therefore more focus has been directed to studying the use of phages as biocontrol agents in food ecosystems (Li *et al*., 2021; Au *et al*., 2022; Lee, Oh and Kim, 2023). There are three possible methods for phage application in food, which can be classified as follows: i) direct application of phages onto food, or onto food processing environment; ii) use of phage lytic enzymes; iii) application phages or their lysins in combination with other antibacterial agents.

2.6.1 Methods of phage application in food systems

There are several critical stages in which bacteriophages can be added to the food product throughout the chain of food process. The understanding of the manufacture process of each food product and the potential source(s) of foodborne pathogens is critical in order to choose the most appropriate bacteriophage application strategy. Studies conducted on phage or their lysins application in food system could be divided into three phage treatment strategies: (i) directly onto ready-to-eat (RTE) foods, or onto the processing environment, (ii) onto raw foods prior to, or during processing and, (iii) in food packaging or utensils.

A large and growing body of literature has investigated the use of phages or the enzymes that they produce as biocontrol agent in RTE minimally processed products such as fruits, vegetables or egg, where heat treatment would be inappropriate (Połaska and Sokołowska, 2019). In addition, the FDA regulations state that RTE foods should be edible and ready for consumption without a final bactericidal processing step to achieve food safety regulations (Food and Drug Administrration, 2016). Therefore, many researchers have applied phages as bactericidal agents against varied pathogens in different products such as: cucumber (Bai, Jeon and Ryu, 2019), Kiwi juice (Thung *et al.*, 2017), packaged lettuce, apples, smoked salmon (Perera *et al.*, 2015), apple juice (Zinno *et al.*, 2014) and cantaloupes (Lone *et al.*, 2016). Although all studies showed a quick reduction from 2 to 5 log CFU/g of target pathogen between 1 and 24 h of surface treatment, complete pathogen elimination was not achieved and

regrowth after around 2 days of storage was observed (Perera *et al.*, 2015; Perera *et al.*, 2015; Bai, Jeon and Ryu, 2019).

A similar criticism was raised by Guenther *et al.* (2012) who tested virulent phage FO1-E2 (10^8 PFU/g) against *Salmonella* Typhimurium (10^3 CFU/g) in a wide variety of RTE food products (hot dogs, cooked and sliced turkey breast, mixed seafood (cooked and chilled cocktail of shrimps, shellfish, and squid), chocolate milk, and egg yolk (pasteurized)). The authors found that pathogen viable count was reduced by 3 log CFU/g after 1 day of storage at 15 °C, however after 2 days, *Salmonella* Typhimurium count increased gradually up to 6 days of storage. Guenther *et al.* (2012) argued that phage lytic ability was not affected, however the ability of phage to diffuse through food matrix to infect target strain was not achievable. Similar observations were also recorded in surface treatment of camembert and Limburger type cheese (Guenther and Loessner, 2011) and queso fresco cheese (Soni *et al.*, 2012). This may encourage researchers to find another method that is not affected by host growth and could work at varied temperature like phage lytic enzyme (endolases or endolysin).

2.6.2 Application of phage lytic enzymes in food systems

Endolysins are alternative antimicrobial agents to viable phages and conventional antibiotics; they are able to directly degrade bonds of peptidoglycan layer of bacterial cell wall (Love *et al.*, 2018). Many studied have investigated the application of endolysins in different food products such as: beef (Chang *et al.*, 2017), soya milk (Zhang *et al.*, 2012) and lettuce (Solanki *et al.*, 2013). In addition, endolysins have been widely tested in various dairy products. Obeso *et al.* (2008) was the first study in testing endolysins antimicrobial activity in dairy environment. They studied the effect of purified endolysin LysH5 at three different concentrations (160, 80 and 45 U/ml) against *S. aureus* Sa9 (10^6 and 10^3 CFU/ml) in whole pasteurized milk. Endolysin LysH5 showed the ability to completely eliminate the host after 4 h of incubation at 37 °C.

Likewise, Chang, Kim and Ryu (2017) reported that a 9 µM endolysin LysSA11 was able to inhibit *S. aureus* (10^3 CFU/ml) after 30 and 60 min of incubation at 25

and 4 °C, respectively, in pasteurized milk. The high endolysin activity was also previously reported in fresh cheese (van Tassell *et al.*, 2017); as well as in milk at combinations with nisin (García *et al.*, 2010; Ibarra-Sánchez, van Tassell and Miller, 2018), bacteriocins (Rodríguez-Rubio *et al.*, 2015) and essential oils (Chang *et al.*, 2017).

Lytic enzymes act as potential antibacterial because of several relevant features, namely their host specificity, rapid antimicrobial effect, decreased probability for target organism resistance, and no transmission of virulence genes (Obeso *et al.*, 2008; Zhou *et al.*, 2017). However, there are some limitations in the usage of phage lytic enzymes; the mode of action of phage lytic enzymes is distinct between Gram-positive and Gram-negative cells. As, Gram-positive cells have an external layer of peptidoglycan, which is easily reachable. However, Gram-negative cells have an external rigid membrane on the peptidoglycan; which acts as an effective barrier to external phage endolysins; that could explain the very limited studies on endolysins application against Gram-negative species. Furthermore, endolysins are higher in cost compared to active phage treatment and high probability to affect natural raw milk microbiota (Oliveira *et al.*, 2012; Cooper, 2016; Love *et al.*, 2018).

2.6.3 Phage application in dairy products

Several studies have achieved a high biocontrol activity in pasteurized milk and in some cheese types, when treated directly in milk at the beginning of manufacture, the results of these studies are summarized in Table 2.4. Of note, it was observed that cheeses studied reached a minimum pH of 5.4 and the maximum salt content was 4 %. However, most cheeses typically have pH value from 4.6 to 6.5 and a salt concentration from 0.7 to 6.0 % (Fox *et al.*, 2017a). Far too little attention has been paid to testing phage efficacy against foodborne pathogens in raw milk, cheeses, and phage application at more challenging physicochemical conditions. In particular, concerns have been raised by several relevant studies on the influence of milk components and technological treatments on phage activity as a biocontrol agent (O'Flaherty *et al.*, 2005a; Gill *et al.*, 2006; García-Anaya *et al.*, 2020).

Table 2.4. Examples of phage application as biocontrol agent in dairy products.

Pathogen (log CFU/ml)	Phage (log PFU/ml)	Product	Time, (temperature)	Outcomes (log CFU reductions)	References
S. aureus DPC5645 (6 log)	K (8 log)	Raw milk	8 h (37 °C)	N.D.	(O'Flaherty *et al.*, 2005a)
S. aureus DPC5645 (6 log)	K (8 log)	HT milk	6 h (37 °C)	7	(O'Flaherty *et al.*, 2005a)
S. aureus Sa9 (2 log)	Cocktail (2 log)	UHT milk	2 h (37 °C)	7	(García *et al.*, 2009)
S. aureus Sa9 (6 log)	Cocktail (8 log)	UHT whole milk	2 h (37 °C)	6	(García *et al*. (2007)
Salmonella LT2 (4 log)	P22 (12 log)	Pasteurized whole milk	48 h (4 °C)	5	(Zinno *et al.*, 2014)
E. coli O127:H6, O5:H (5 log)	Cocktail (9 log)	UHT milk	3 h (25 °C)	5	(McLean, Dunn and Palombo, 2013)
L. monocytogenes (3 log)	A511 (7 log)	Chocolate milk	6 days (6 °C)	5	(Guenther *et al.*, 2009)
S. aureus Sa9 (6 log)	Cocktail (6 log)	Fresh cheese	6 h (37 °C)	6	(Bueno *et al.*, 2012)
Salmonella Enteritidis (4 log)	SJ2 (8 log)	Chedder cheese	2 h (37 °C)	4	(Modi *et al.*, 2001)
S. aureus Sa9 (6 log)	Cocktail (6 log)	Hard cheese	24 h (32 °C)	5	(Bueno *et al.*, 2012)
E. coli RT1 (6 log)	Cocktail (7 log)	Semi-hard cheese	60 days (4 °C)	6	(Tabla *et al.*, 2022)

2.6.3.1 Challenges of phage application in dairy environment

The mechanism involved in the phage infection process in complex systems such as milk and dairy products still need to be clarified (García-Anaya *et al.*, 2020). In this section, the accompanied chemical, microbial and technological aspects that may affect phage biocontrol activity are summarized.

2.6.3.1.1 Milk composition and structure

In general, cow's milk is composed of 87.7 % water, 4.9 % lactose (carbohydrate), 3.4 % fat, 3.3 % protein, and 0.7 % minerals (referred to as ash). The quantities of the main milk constituents can vary considerably between species (cow, goat, sheep), breed (Holstein, Jersey), animal's feed, and the stage of lactation (Fox *et al.*, 2017b).

About four fifths of the protein consists of casein (79.5 % of total protein), that is composed of α_{S1}-, α_{S2}-, β-, and K-casein. In addition, whey proteins (milk-serum proteins; 20.5 % of total protein) are mixture of α-lactalbumin, β-lactoglobulin, blood serum albumin, immunoglobulins and miscellaneous (including proteose-peptone) (Fox, 2003). Casein is present in a caseinate form, which means that it binds cations, primarily calcium and magnesium. Furthermore, the electrochemical status of caseins is obtained from net charge in the side chains of some amino acids, which is determined by the pH of the milk. At pH 6.6 (normal milk pH), casein protein has a net negative charge, where protein molecules remain separated because of the repulsion force between identical charges (Fox *et al.*, 2017b).

On the other hand, milk fat consists of triglycerides, di- and monoglycerides, fatty acids, sterols, carotenoids and soluble vitamins (A, D, E, and K), while the membrane of milk fat globules consists of phospholipids, lipoproteins, cerebrosides, but also contains proteins, nucleic acids, enzymes and trace elements. Milk also contains many trace elements of minerals (K, Na, Ca, Mg and Cl), enzymes and leukocytes (Fox, 2003).

All mentioned non-target components would mainly affect the probability of phage to get in contact with the target bacterial cell, by acting as a physical barrier between phage a

2.6.3.1.2 Effect of technological variations on milk components

In the dairy industry, there is a strong relationship between milk physical, chemical and biochemical phenomena and process operation and design. There are some main technological treatments that affect significantly the natural milk composition or characteristics (Hansen, Larsen and Wiking, 2021), that could subsequently affect phage biocontrol activity (Gill *et al.*, 2006; García-Anaya *et al.*, 2019).

The applied heat treatments in the dairy industry can be divided into four types: thermalization (65 °C, 20 sec; for inactivation of psychrotrophic bacteria), high-temperature-short-time (HTST) pasteurization (74 °C, 20 sec; for inactivation of pathogenic bacteria), high pasteurization (85 °C, 20 sec; for inactivation of all microorganisms but not spores) and sterilization or ultra-high-temperature (UHT) treatment (110 – 140 °C, 5 - 10 sec; to destroy spores) (Britz and Robinson, 2008). The heat-induced changes in milk could be summarized to main effects on destruction of microorganisms, inactivation of milk enzymes, and mainly denaturation of proteins. Denaturated whey proteins had different structure as shown in Figure 2.4c, where change in protein structure would influence negatively or positively many functional properties of dairy products. As denaturated whey protein is precipitated on casein micelle, which increase the water binding capacity of proteins (Britz and Robinson, 2008). This justify the cheese producer's preference to low or no heat treatment during artisanal cheese manufacture (Little *et al.*, 2008). In addition, part of the associated whey proteins are the immunoglobulins, where the denaturation of mainly IgA and IgM, would suppress milk fat agglutination and aggregation of fat globules (D'Incecco *et al.*, 2018).

Changing from milk liquid state to solid or gel state after coagulation may also affect phage lytic activity and movement as previously described. However, an understanding of the differences between each coagulation type would help to explain data variability. In particular, acid coagulation is occurred after decreasing the milk pH close to casein iso-electric point (IEP; 4.5 to 4.9), at this stage the colloidal calcium (in casein micelle), will dissolve and form ionized calcium and due to removal of repulsion force (casein net charge = 0), casein protein only will precipitate and form acid curd. nd bacteria (Greer, 2005).

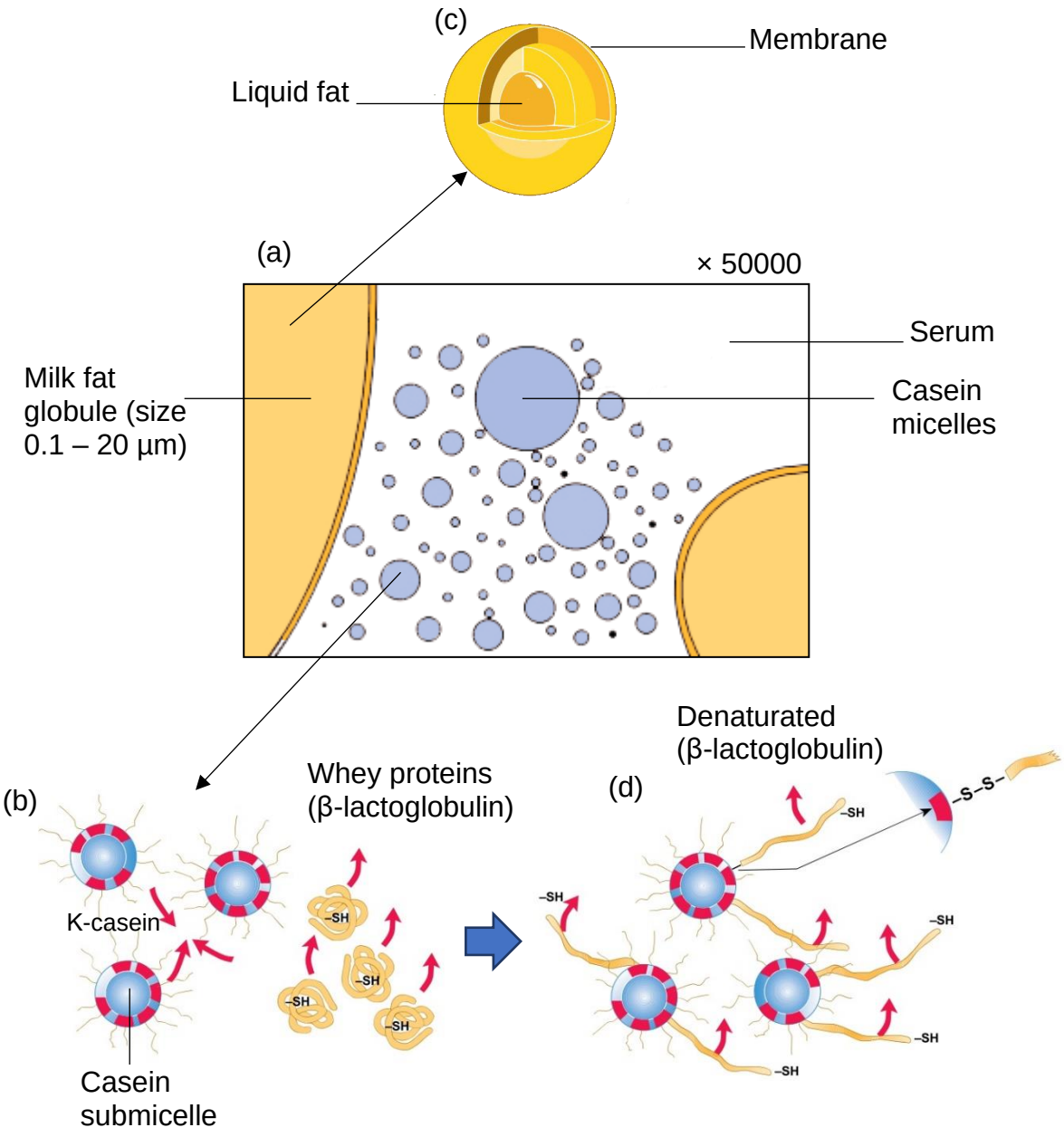

Figure 2.4. Milk view at × 50000 magnification (a) with casein, whey protein (b) and fat globule (c) structure, in addition to the effect of heat treatment on whey proteins (c). Images adopted and modified from Tetra Pak (1995) and Smith, Cook and Smith (2001).

As there is no bonding between casein micelles, the resultant curd will be porous and have lower water binding capacity due to whey protein losses in whey (Tetra Pak, 1995; Fox *et al.*, 2017c). On the other hand, enzymatic coagulation occurs by using proteolytic enzymes (rennet or chymosin) that attack the K-casein and split to glycomacro-peptide and para- K-casein, leaving the present α- and β-casein to bind together by calcium ions and form the enzymatic curd. Accordingly, the enzymatic curd would be more rigid and has higher water binding capacity.

2.6.4 Commercial bacteriophage products

The growing interest in using phages as biocontrol agents has led to the manufacture of phage-based products by various companies worldwide. A number of phage-based preparations have passed regulations to be used in food processing and agriculture, including Canada, the USA, Switzerland, Australia, and New Zealand (Au *et al.*, 2022). While currently phage products are not regulated to be used within the European Union and United Kingdom, they are allowed only on food that will be exported to countries where it is authorized. Interestingly, the companies involved in production and commercialization of phage-based products as biocontrol method are increasing in number (Gildea, Ayariga and Roberson, 2022).

The United States Food and Drug Administration (USFDA) and United States Department of Agriculture (USDA) have approved several phage-based preparations, such as:

(i) ListexP-100™, a commercially available bacteriophage preparation, manufactured by Micreos (EBI Food Safety, Wageningen, the Netherlands) and generally recognized as safe (GRAS) for all food products. Many researchers have tested the efficiency of bacteriophage P-100 for controlling *Listeria monocytogenes* in different food products, such as raw salmon (Baños *et al.*, 2016), UHT whole milk (Komora *et al.*, 2020) and queso fresco cheese (Soni *et al.*, 2012).

(ii) ListShield™ (formerly LMP-102)

(iii) SalmoFresh™

(iv) Salmonelex™

(v) EcoShield™ are manufactured by Intralytix Ltd (United States of America).

These preparations are designed to control foodborne bacterial pathogens such as *Listeria monocytogenes, Salmonella* and *Escherichia coli*, respectively (Carter *et al.*, 2012; Moye, Woolston and Sulakvelidze, 2018). Interestingly, some of the phage products were also certified as Halal and Kosher (Moye, Woolston and Sulakvelidze, 2018).

2.7 Summary

There is significant knowledge available on the emergence of foodborne pathogens in raw milk cheeses especially those manufactured under uncontrolled hygienic conditions. However, to date there is no ideal antibacterial agent that could be applied without compromising raw milk cheese manufacturing logistics and characteristics. Studies on phage application in milk and some dairy products showed the potential of future phage success as biocontrol agent in raw milk cheeses. Particularly, researchers recommended phage application in milk instead of cheese. So far, however, there has been little discussion about the effect of milk components on phage performance against pathogens, and there is no clear understanding and contradictory views on this mechanism. In addition, little or no research has investigated the behaviour of phages under a wide range of cheese related physicochemical conditions, and their application in raw milk hard and pickled cheese, therefore further research is required to reach a better understanding of phage success as biocontrol agent in artisanal cheeses.

Chapter Three: Materials and Methods

3.1 Research Experimental Design

An experimental design procedure was developed to identify the efficacy of selected *E. coli* and *S. aureus* phages to enhance the safety of raw milk Egyptian cheese; that provide more understanding of the phage behaviour in dairy environment with varied physicochemical properties. The experimental design includes five phases as described in Table 3.1, to examine the potential and limitations of bacteriophages for controlling *E. coli* and *S. aureus* strains during manufacture and storage of three different types of Egyptian cheeses (Karish, Domiati and Ras) with unique physicochemical properties.

The study design started with isolation of *E. coli* and *S. aureus* strains from cow's milk (phase 1; Chapter 4), to identify the ability of the obtained phages to target dairy origin hosts (phase 3; Chapter 6). Thereafter, the manufacture of Karish, Domiati and Ras cheeses (phase 2; Chapter 5) was essential to simulate the Egyptian cheese manufacture in small scale laboratory conditions; and identify the critical physicochemical conditions that phages will be exposed to during cheese manufacture and storage (Table 3.1).

Table 3.1. Details of the research experimental design followed in this study.

Research phase	Aim	Analytical steps	Chapter
Phase 1	Isolate and identify *E. coli* and *S. aureus* strains from raw milk.	Isolates were analysed using: a) Morphologic and growth characterisation; b) *E. coli* biochemical characterisation: Urease activity, Oxidase activity and API 20 E; c) *S. aureus* biochemical characterisation: Gelatine hydrolysis, catalase test, coagulase test and API Staph; d) Molecular characterisation and PCR amplification.	4
Phase 2	Identify the critical physicochemical conditions of cheese in small scale laboratory conditions.	Karish, Domiati and Ras cheese samples were analysed during manufacture and storage for fat %, total protein %, salt %, moisture %, pH, water activity and texture profile analysis.	5
Phase 3	Characterise four *E. coli* and four *S. aureus* phages to select the ideal phages as biocontrol agent in Egyptian raw milk cheeses.	a) Determine the minimum phage concentration required to inhibit the host strain using 10^3 to 10^6 PFU/ml phage against 10^3 to 10^6 CFU/ml host; b) Evaluate phages performance under previously identified physicochemical conditions of temperature (45, 37, 24 and 4 °C), pH (6.7, 5.9, 5.1 and 4.2) and salt (0.5, 4, 8, 12 %); c) Determine phage host range using isolated and reference strains.	6
Phase 4	Evaluate the effect of raw milk-associated microorganisms and skimming of milk fat on selected *E. coli* and *S. aureus* phage lytic activity.	a) Test phage (10^5, 10^6 and 10^8 PFU/ml) lytic ability in sterilized whole and skimmed milk; b) Test phage (10^8 PFU/ml) lytic ability in raw whole and skimmed milk; c) Test phage adsorption rate in sterilized and raw (whole and skimmed) milk.	7
Phase 5	Quantify the *in vivo* efficiency of phages to control pathogen growth during cheese manufacture and storage.	Monitor the influence of selected *E. coli* and *S. aureus* phages (10^8 PFU/ml) as biocontrol agent in Karish, Domiati and Ras cheese by testing total bacterial count, *E. coli* or *S. aureus* count and phage titre.	8

That leads to phase 3 (Chapter 6) which focused on: firstly, determine the ideal phage concentration with highest lytic ability to the host strain for more cost and time efficiency; and secondly, test the impact of Egyptian raw milk cheese physicochemical conditions on *E. coli* and *S. aureus* phage's lytic ability and host strain growth rate. Based on the understanding of the four *E. coli* and four *S. aureus* phages characterisation (phase 3), the ideal phages with respective concentration that have high potential success as a biocontrol agent in cheese application were selected. The selected phages were used to test phage-mediated reduction of *E. coli* and *S. aureus* in raw cow's milk (phase 4; Chapter 7) prepared to be applied in Egyptian raw milk cheeses (phase 5; Chapter 8) (Table 3.1).

3.2 Small Scale Manufacture of Egyptian Raw Milk Cheeses

The main cheese manufacture technique was followed to: firstly, determine and monitor Karish, Domiati and Ras cheeses physicochemical conditions during manufacture and storage (section 6.3.2) and secondly, *in vivo* *E. coli* and *S. aureus* phage application in Karish, Domiati and Ras cheese to control pathogen growth (Chapter 8).

Three types of Egyptian cheeses (Karish, Domiati and Ras) were manufactured in a category two containment microbiological laboratory, at Harper Adams University (HAU), United Kingdom. The Egyptian cheeses were manufactured from raw cow's milk obtained from HAU bulk tank and transferred to the laboratory within 10 min, and then the raw milk chemical composition (total solids, solids-not-fat, fat and lactose) was analysed by MilkoScan (MilkoScan™ Minor 78110, FOSS, Denmark) in duplicate. The pH and temperature were measured in milk and during cheese manufacture and storage using a portable electrode for cheese analysis (HANNA HI-99165, UK).

3.2.1 Karish cheese

The collected raw cow's milk (10 Litres) (section 3.2) was transferred to a cheese vat (BUFFALO L310-02, UK) for temperature adjustment to 40 °C prior to fat separation by using cream separator (Milky FJ 90 PP, Austria). The fat percentage of the raw cow's skimmed milk was standardised at 0.5 % by double milk separation. Karish cheese was then manufactured from collected raw skimmed milk (RSM) as described by Allam *et al.* (2017) (Figure 3.1). The RSM temperature was adjusted to 37 °C and addition of 0.1 % YO-LP freeze-dried starter culture (Proquiga, Spain) to reach complete acid coagulation after 18 h. The curd formed after 18 h was then transferred to cheese cloth 600 mm (24'') square (GN Ltd, UK) and left for 18 h with light pressing to enhance whey separation. A 1 % (of cheese weight) dry cheese salt (GN Ltd, UK) was added and left for another 2 h for salt absorption and further whey separation. Finally, the cheese was vacuum packaged (150 × 350 mm) using portable vacuum pack machine (BUFFALO, UK) and stored at 4 °C for 15 days.

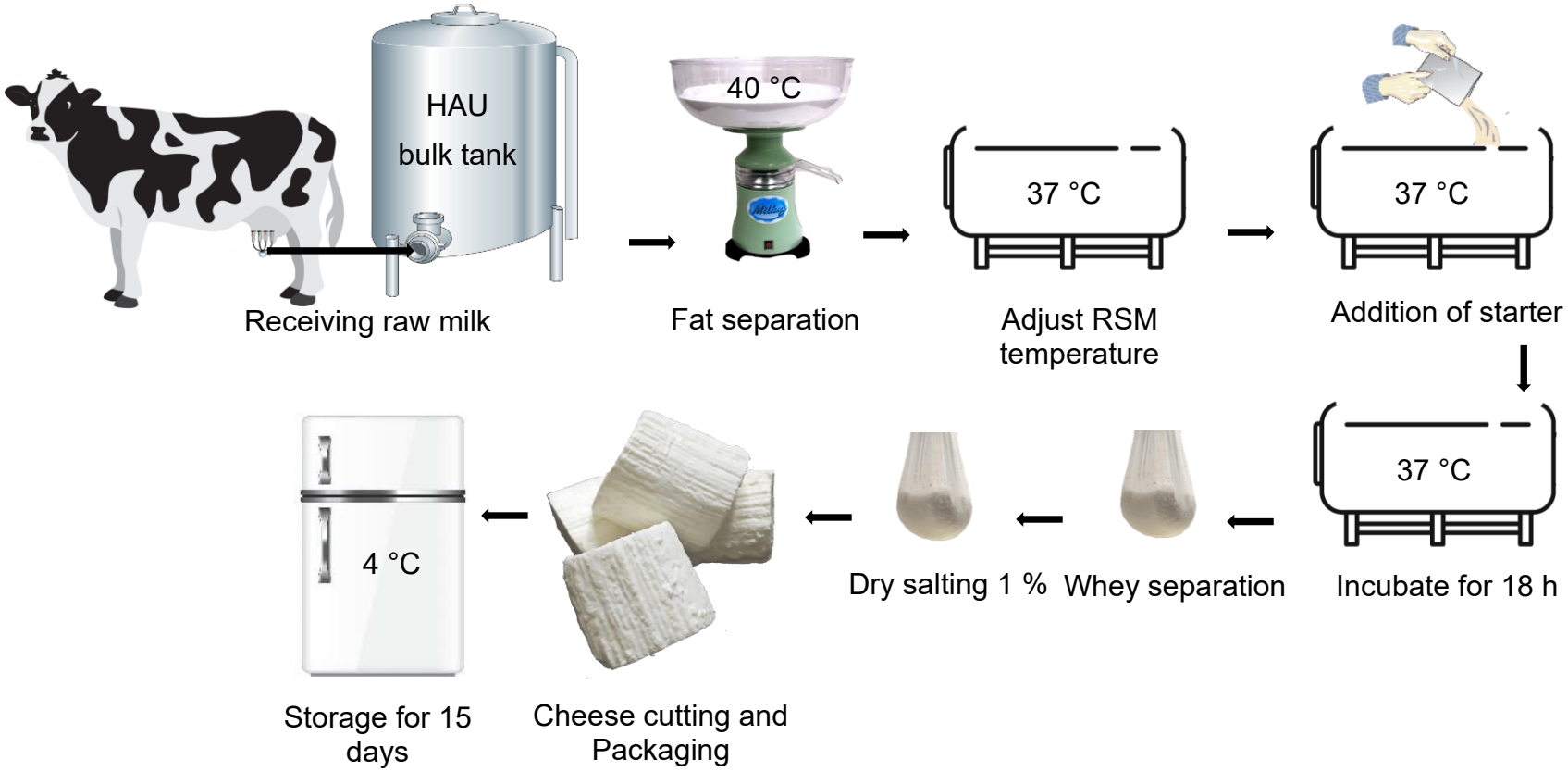

Figure 3.1. Flow diagram showing manufacture process of Karish cheese.

3.2.2 Domiati cheese

Domiati was manufactured as described by Ayad (2009) (Figure 3.2). The collected raw cow's milk (10 Litres) (section 3.2) was transferred to cheese vat (BUFFALO L310-02, UK) for temperature adjustment to 37 °C. After that dry cheese salt (8 % from milk volume) was mixed well with raw cow's milk at the beginning of the manufacture. The manufacturer's recommended amount of animal rennet (145 IMCU/ml) (GN Ltd, UK) was doubled (~10 ml) because of the reduced rennet activity due to high salt content. Once the raw milk was enzymatic coagulated in 3 h, the curd was transferred to cheese cloth 600 mm (24'') square (GN Ltd, UK) and left for 18 h with medium (~2 kg) pressing to enhance whey separation. The cheese was then cut into cubes (around 400 mm × 600 mm) and stored in brine (10 % salt) solution at 4 °C for minimum 30 (Chapter 8) to 90 (Chapter 5) days.

3.2.3 Ras cheese

Ras was manufactured as described by Awad, Ahmed and Soda (2007) (Figure 3.3). The collected raw cow's milk (20 litres) (section 3.2) was transferred to cheese vat (BUFFALO L310-02, UK) for temperature adjustment to 32 °C. A 0.1 % YO-LP starter culture (Proquiga, Spain) was added and left for 1 h at 32 °C to enhance acid production during scalding and ripening development. In addition, annatto-food-colouring (0.5 ml per 1000 ml) (GN Ltd, UK) was added to give Ras cheese its distinctive yellow colour. The manufacturer's recommended amount of animal rennet (~10 ml; 145I MCU/ml) (GN Ltd, UK) was added to clot the milk in 30 to 40 min. The curd was cut into cubes (~2 cm^2) by curd cutter (GN Ltd, UK) and formed curd cubes were allowed to rest in the whey for 5 to 10 min. After that, the curd was scalded for 45 min by raising the temperature gradually to 45 °C and then holding this temperature for a minimum of 30 min with continuous manual stirring. The whey was then drained when the curd pH value reached 5.9. The obtained curd was hooped in hard cheese mould (90 x 110 x 110 mm), pressed at 20 lb for 4 h, and then the cheese was pressed at 50 lb for 18 h. The edges of cheese block were trimmed, then the cheese block was inverted and pressed again at 50 lb for 2 h.

Ras cheese blocks were stored at 9 – 12 °C and 85 % relative humidity (RH) for minimum 90 (Chapter 8) and 180 (Chapter 5) days. The temperature and relative humidity (RH) were measured during storage of cheese by HOBO data logger (HOBO MX1101, UK). Salting was done in two stages: firstly, when curd was still in the whey; secondly, dry cheese salt was spread on top of cheese and after two days cheese block was turned upside down and the process repeated up to 12 days in order to obtain 3.5 – 4.0 % salt content in cheese. Ras cheese was waxed after 15 days of ripening by brushing cheese blocks with clear cheese coating (GN Ltd, UK) and lifted to dry for 2 h.

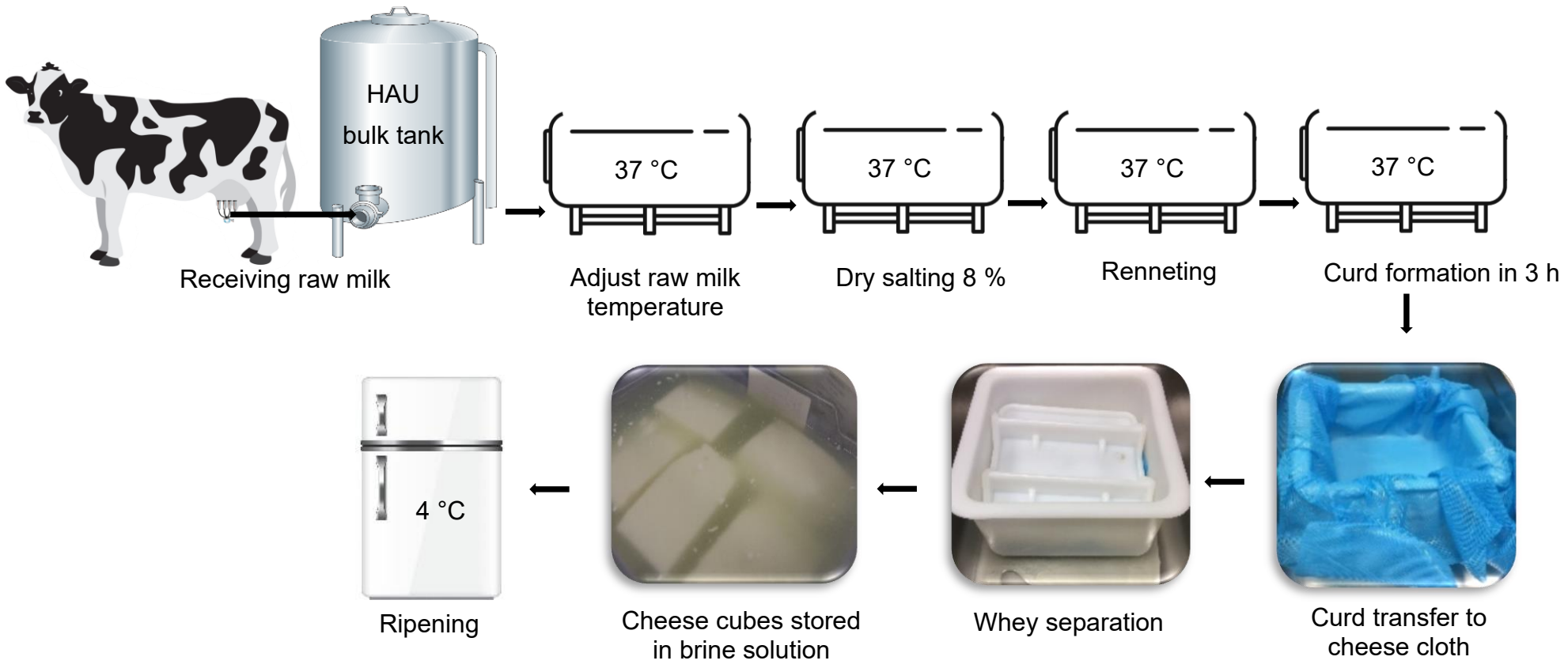

Figure 3.2. Flow diagram showing manufacture process of Domiati cheese.

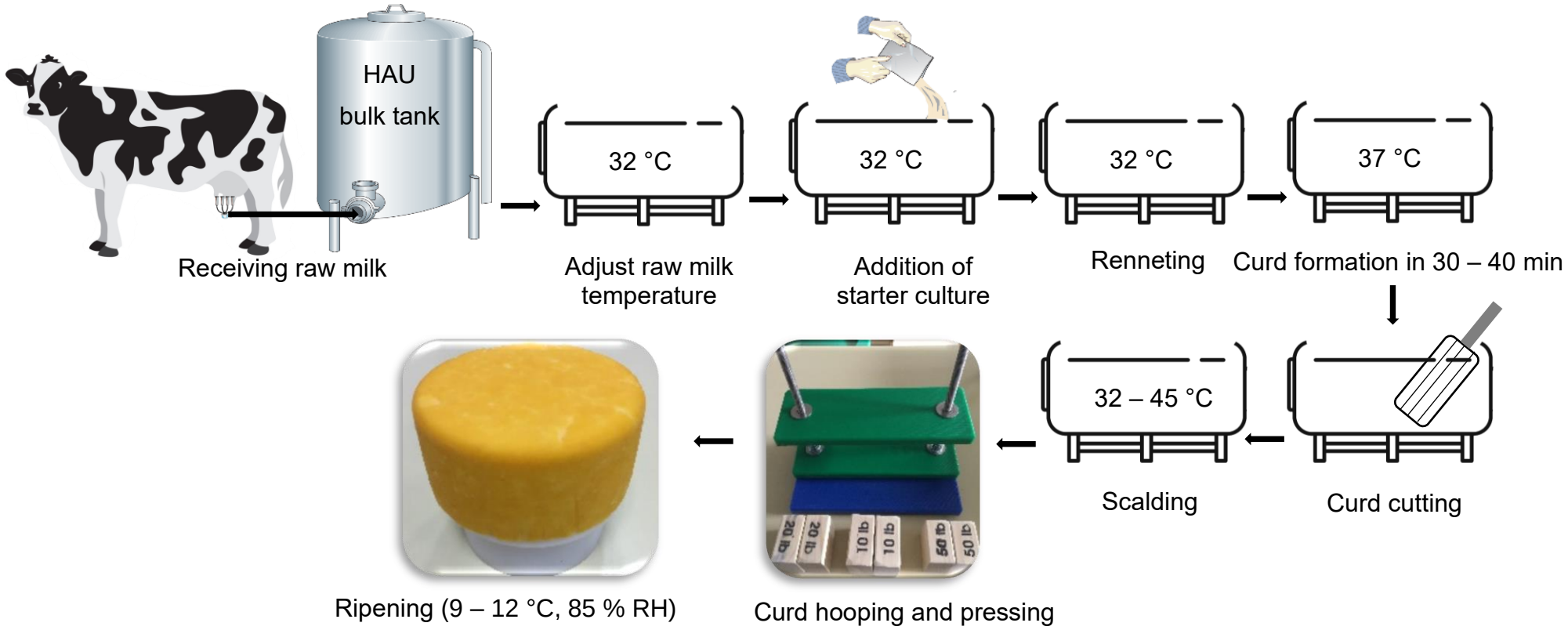

Figure 3.3. Flow diagram showing manufacture process of Ras cheese.

3.3 Bacterial Strains and Culture Conditions

Bacterial strains were obtained in freeze-dried or liquid form from the German Collection of Microorganisms and Cell Cultures (DSMZ), American Type Culture Collection (ATCC), National Collection of Type Cultures (NCTC) and National Collection of Industrial Food and Marine Bacteria (NCIMB). Bacterial stains were used in the following experiments as: a) a quality control cultures for *E. coli* and *S. aureus* identification; b) phage host strains; and c) target strains to test phage host range. The available bacterial strains from dairy or food environment were selected; however, other environmental and clinical isolates were also used (Table 3.2). According to United Kingdom regulations, any work on *E. coli* O157-H7 must to be established in a category three containment laboratory, because STEC *E. coli* is considered as a hazard group 3 organism in UK (NHS, 2015). Due to the inaccessibility of category three containment laboratory in HAU, this study was performed on generic *E. coli* and *S. aureus* strains.

In addition, five lactic acid bacterial (LAB) isolates (Awad, Ahmed and Soda, 2007) from Egyptian dairy products (donated by Laboratory of Dairy Microorganisms and Cheese Research, Alexandria University) were used in phage host ranges testing.

Bacterial strain cultures were stored at -80 °C in protect micro-organism preservation beads in cryovial (TSC, UK); and were also streaked on slant Trypticase Soy Agar (TSA; LAB M., UK). Slants were re-streaked after 1 month of storage at 4 °C.

3.3.1 *Escherichia coli* strains

All *E. coli* strains (Table 3.2) were streaked from frozen cultures on MacConkey agar medium (LABM, UK) with incubation at 37 °C for 24 h and Gram stained. Pink colonies were streaked on Eosin Methylene Blue Agar (EMB; LABM, UK) and then stored at 4° C, until further use for not more than 2 days.

Table 3.2. Details of bacterial strains used in this study.

Strain name	Strain ID	Origin of isolation
Non-pathogenic *E. coli* surrogate	ATCC BAA-1427	Cattle Hides
Non-pathogenic *E. coli* surrogate	ATCC BAA-1428	Cattle Hides
Non-pathogenic *E. coli* surrogate	ATCC BAA-1429	Cattle Hides
Non-pathogenic *E. coli* surrogate	ATCC BAA-1430	Cattle Hides
Non-pathogenic *E. coli* surrogate	ATCC BAA-1431	Cattle Hides
E. coli	DSM 22665	Mastitis Milk
E. coli	DSM 101113	Clinical Isolate
E. coli	DSM 106579	Human Blood
E. coli	NCIMB 10243	Faeces
S. aureus	DSM 104437	Clinical Isolate
S. aureus	DSM 105272	N.A.
S. aureus	DSM 12463	Mastitis Milk
S. aureus	ATCC 33591	N.A.
S. aureus	ATCC 25923	Clinical Isolate
S. aureus	ATCC 19685	Clinical Isolate
S. aureus	NCTC 13552	Bulk Milk
S. warneri	DSM 20036	Clinical Isolate
S. epidermidis	DSM 28319	Clinical Isolate
S. haemolyticus	DSM 20263	Human Skin
S. hyicus	DSM 17421	N.A.
E. hermanni	DSM 4560	Human Skin
K. pneumoniae	DSM 789	Cow's Milk
Y. enterocolitica	DSM 11067	N.A.
L. monocytogenes	DSM 15675	Soft Cheese
B. coagulans	DSM 1	Evaporated Milk
L. helveticus	27 st	Ras Cheese
Lb. paracasei subsp. *paracasei*	119 th	Domiati Cheese
En. faecium	102 E	Ras Cheese
En. faecium	236 E	Ras Cheese
En. faecium	241 E	Ras Cheese

*N.A.: Not available

3.3.2 Staphylococcal spp. strains

All Staphylococcal strains (Table 3.2) were streaked from frozen cultures on Baird-Parker agar medium (LABM, UK) with 5 % egg yolk tellurite (LABM, UK) with incubation at 37 °C for 24 h and Gram stained. Based on distinct morphological characteristics and formation of opaque halos, colonies were selected for further analysis. *S. hyicus*, *S. epidermidis*, *S. haemolyticus* and *S. warneri* were used in phage host ranges testing.

3.3.3 Other pathogenic strains

Klebsiella pneumoniae, *Yersinia enterocolitica*, *Listeria monocytogenes* and *Bacillus coagulans* strains (Table 3.2) were streaked from frozen cultures on Trypticase Soy Agar (LABM, UK) with incubation at 30 °C for 24 h, and then stored at 4 °C and Gram stained. Those strains were used in phage host ranges testing.

3.3.4 Lactic acid bacteria strains

Lactobacillus helveticus, *Lactobacillus paracasei* subsp. *paracasei* and *Enterococcus faecium* strains (Table 3.2) were streaked from frozen cultures on de Man, Rogosa and Sharpe (MRS) Agar (MRS; LABM, UK) with incubation at 37 °C for 24 h and Gram stained, and then stored at 4 °C. Those strains were used in phage host ranges testing.

3.3.5 Gram stain protocol

Bacterial strains were Gram stained as described by Smith and Hussey (2019). A single colony from each streaked agar plate according to tested species, was spread on clean, grease free slide then air-dried and heat-fixed. The slide with smear of cells was dipped for 1 min in crystal violet staining reagent (Fisher, US). The slide was then washed for 2 sec by distilled water and dipped in Gram's iodine (Fisher, US) for 1 min. After that, the slide was washed by distilled water and dipped for 15 sec in ethyl alcohol 95 % (vol/vol) (Sigma Aldrich, Germany) with repeat washing. The slide was then dipped in safranin (Fisher, US) for 30 sec and washed gently for 2 sec by distilled water. Finally, the

resulted slide was left to dry and observed by oil immersion using a Brightfield microscope at 1000x magnification with oil immersion. Gram-negative bacteria were stained pink or red and Gram-positive bacteria were stained blue or purple.

3.4 Bacteriophages and Culture Conditions

Four *E. coli* and four *S. aureus* phages were obtained in freeze-dried or liquid form from the German Collection of Microorganisms and Cell Cultures (DSMZ), American Type Culture Collection (ATCC) and National Collection of Industrial Food and Marine Bacteria (NCIMB). The origin and respective phage host strain used in this study are described in Table 3.3.

Phage selection was made according to mainly, the most reported phages in literature (for *E. coli* phage T4 and *S. aureus* phage K) and other phage sources were also checked, however due to fixed availability and limitations at the time, the other phages were selected. It was previously reported that EcoShield phage (Intralytix, USA) is able to target non-pathogenic *E. coli* surrogates (Table 3.2; Stratakos and Grant, 2018), however it was not achievable to use this phage mixture in this study.

Phages were stored in 1 ml Saline-Magnesium (SM) buffer containing 50 % glycerol (Fisher, US); the mixture was then mixed well and placed in -80 °C freezer (García *et al.*, 2009). The SM buffer [0.05 M Tris-HCl (Sigma Aldrich, Germany), 0.1 M NaCl, 0.008 M $MgSO_4$ (Fisher, US) and pH adjusted to 7.5] ensured that essential electrolytes for effective phage adsorption, replication (Bandara *et al.*, 2012) and subsequent death of target bacterial cell (García *et al.*, 2010) were present.

Table 3.3. Details of phages and host strains used in this study.

Phage name	Phage ID	Host strain ID	Origin of isolation
E. coli T4	NCIMB 10360	NCIMB 10243	Sewage
E. coli K1E	DSM 101793	DSM 101113	Surface water
E. coli EcoS	DSM 101792	DSM 101113	Sewage
E. coli EcoM	DSM 106580	DSM 106579	Sewage
S. aureus EBHT	DSM 26856	DSM 104437	Horse nostrils
S. aureus CS1	DSM 105264	DSM 105272	Pig snout
S. aureus K1	DSM 26857	DSM 104437	Farmyard slurry
S. aureus K2	ATCC 19685	ATCC 19685	N.A.

*N.A.: Not available

To prepare broth overnight culture of host strains for phage testing, each bacterial host strain was streaked on a Nutrient agar (NA) plate (LAB M., UK) and incubated at 37 °C for 24 h. A single colony from each NA-streaked plate was then inoculated into Luria Bertani Broth (LB; Fisher; US) and incubated at 37 °C for 24 h.

3.4.1 Bacteriophage stocks preparation

The high-titre phage suspensions were prepared as described by Sambrook and Russell (2011); to obtain sufficient volumes and titres (10^9 - 10^{11} PFU/ml) of *E. coli* and *S. aureus* phages for phage analysis (Chapters 6, 7 and 8).

A 100 µl aliquot of frozen phage culture (1/10) was mixed with 100 µl of overnight host strain culture. A control of phage-only and host-strain-only were prepared. The treated and control cultures were incubated at 37 °C for 20 min (to allow phage particles to adsorb to the bacteria). After that, 4 ml of molten LB agarose overlay [SM buffer, 0.025 % LB broth powder (Fisher, US), 0.5 % agarose (Sigma Aldrich, Germany)] (47 °C) was added to the culture tube, then mixed gently and poured directly to LB agar plate ensuring an even distribution on the plate. The plates were left for 5 min to hardened and were then incubated

at 37 °C for 24 h. After incubation, 5 ml of SM buffer was added to each plate (showing complete lysis) and then stored at 4 °C for 24 h.

The SM buffer was then collected using 5 ml luer lock syringe (Fisher scientific, Germany). Instead of using chloroform, collected SM buffer was filter sterilised using sterile disposable Millipore filters 0.2 µm (Sarstedt, Numbrecht, Germany) to eliminate bacterial cells; as some *Myoviruses* may be sensitive to chloroform (Ackerman, 2009). The filter sterilized SM buffer was transferred to polypropylene centrifuge tubes and centrifuged at 5800 rpm (3-16PK Sigma, Germany) at 4 °C for 10 min. The supernatant was then filter sterilised again using Millipore filters 0.2 µm (Sarstedt, Numbrecht, Germany), and transferred to 25 ml sterile polypropylene tube (Sarstedt, Numbrecht, Germany). The tube was then wrapped in aluminium foil and stored at 4 °C for further analysis.

The concentration of the prepared phage stock was measured using overlay plaque assay. The main advantages of this method were that: firstly, agarose was preferred to agar because agarose prevents cross-contamination among plaques between different phages (Sambrook and Fritsch, 1989); secondly, small volume of phage was required for propagation.

3.4.2 Overlay plaque assay

The overlay plaque assay technique was used to determine the phage titre of: a) prepared phage stock (section 3.4.1); b) treated milk samples (Chapter 7); and c) treated Karish, Domiati and Ras cheese samples (Chapter 8). Prepared phage samples were enumerated using the overlay plaque assay as described by Sambrook and Russell (2011).

Phage samples were 10-fold serially diluted (from 10^{-1} to 10^{-10}) in SM buffer. After that, a 100 µl aliquot of each phage serial dilution was mixed in a sterile glass tube with 100 µl of an overnight culture of the host strain, and placed on ice (to ensure accuracy of each dilution result). When all tubes of each dilution had been prepared, all tubes were then incubated at 37 °C for 20 min. The mixture was then mixed with 4 ml of molten LB agarose overlay and immediately poured onto LB agar plates (Fisher; US).

A host-strain-only control and phage-only control were made in order to evaluate the results and ensure purity of phage stock.

After incubation at 37 °C for 24 h, the clear plaques (Figure 3.4) were counted and PFU/ml calculated.

(PFU/ml = plaques per plate × volume plated in ml × dilution factor).

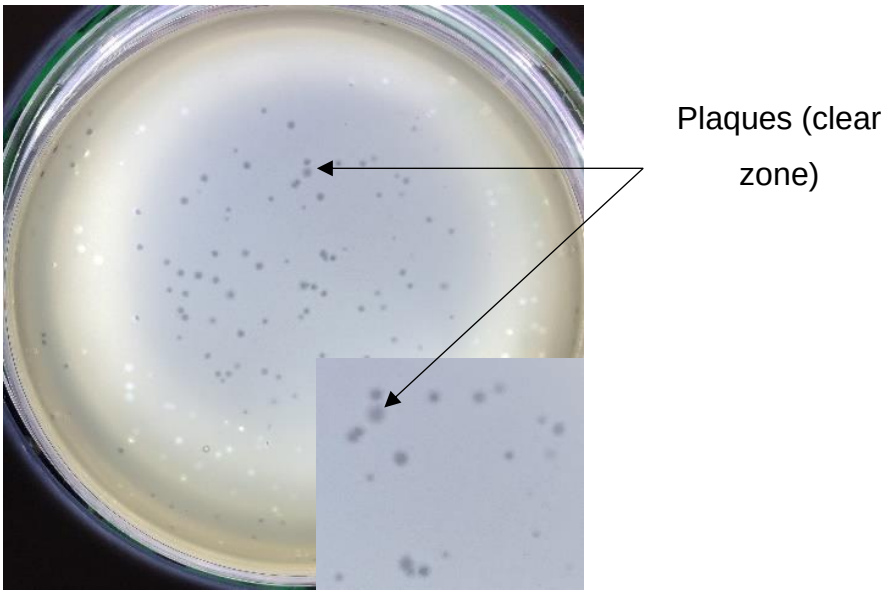

Figure 3.4. Example of the achieved plaque shape in overlay plaque assay test.

3.4.3 Bacteriophage long term storage

The long-term storage of prepared bacteriophage stocks was performed as follows: a) an 0.2 ml of prepared bacteriophage stock (section 3.4.1) for each individual phage was mixed well with 0.2 ml glycerol (Fisher, US) in 2 ml vials to give final 50 % concentration of glycerol (García *et al*., 2009); b) the mixture was then placed overnight in -80 °C freezer; c) the prepared vials were subjected to freeze-drying in freeze-drier (Labconco, Kansas City, MO) for 30 h at 0.6 mbar chamber pressure; c) after complete freeze-drying, vials were stored at -80 °C.

Chapter Four: Isolation of *E. coli* and *S. aureus* Strains from Raw Cows' Milk

4.1 Introduction

As discussed in Chapter 2, *E. coli* and *S. aureus* are the most reported pathogenic bacteria in Egyptian raw milk cheeses (Ombarak *et al*., 2016; Kamal *et al*., 2017; Abdel-Hameid *et al*., 2019; Zayda *et al*., 2020). These microbial pathogens get access to milk either by direct secretion from udders with clinical or subclinical mastitis or by contamination from the environment during handling of raw milk (Rosengren *et al*., 2010; Ombarak *et al*., 2016).

As the research aim is to control *E. coli* and *S. aureus* in Egyptian raw milk cheeses using bacteriophages under small scale laboratory conditions, the isolation and identification of foodborne pathogens obtained from milk samples is a substantial aspect for pathogen control programs (Hu *et al*., 2016; Baños *et al*., 2016). Many researchers reported that phage lytic activity could be affected positively or negatively by the origin of target strain due to variation in their susceptibility to antimicrobial treatments or stressful conditions (O'Flaherty *et al.*, 2005b; Hudson *et al*., 2005; Horlbog *et al.*, 2018). For example, O'Flaherty *et al.* (2005b) found that *S. aureus* phages CS1 and DW2 had stronger lytic ability against *S. aureus* strains isolated from farm environment compared to clinical isolates.

In addition, phages are highly specific for host bacteria within the same species or across related species, a characteristic known as host range. This host specificity is one of the features whereby phages can be classified (Chapter 2; Simmonds and Aiewsakun, 2018). Phage infection of bacteria begins with adsorption of phage particles to specific receptor sites on the host cell surface

(Fister *et al.*, 2016a); bacterial strains isolated form different regions have genetic and physiological differences, which leads to changes in phage receptors and consequently, ability to target specific strain (O'Flaherty *et al*., 2005a; García *et al*., 2009; Porter *et al*., 2016). Therefore, it is necessary to test phages against pathogens isolated from the same source of intended application. Notably, Duffy, Grau and Vanderlinde (2000) reported that generic *E. coli* strains could be used in challenge studies for the validation of designed protocol for pathogenic *E. coli* control.

Therefore, the objective of this work (phase 1, section 3.1) is to isolate generic *Escherichia coli* and *Staphylococcus aureus* strains from raw milk to create a large culture collection to be used as host strains for bacteriophage host ranges testing (section 6.2.2.6).

4.2 Experimental Setup

4.2.1 Study design

This study was designed to isolate and identify *E. coli* and *S. aureus* strains from cows' milk at Harper Adams University (HAU) dairy unit. A total of 100 raw cows' milk samples were collected during the period between June 2018 and October 2019, using the following methods as described in Figure 4.1. The isolation protocol was performed on individual cow's milk samples after pre-enrichment by incubating raw milk samples at 37 °C for 24 h. According to Artursson, Nilsson-Öst and Persson Waller (2010) specific enrichment for milk samples should be performed to enhance colonies appearance and count in tested samples; these findings were also reported by Kreausukon *et al.* (2012) and Miszczycha *et al.* (2016).

4.2.2 Sampling & data collection

Cows producing milk with the highest total bacterial count were selected according to the microbiological data available from the HAU dairy unit. This selection was done in order to increase the probability of *E. coli* or *S. aureus* presence in selected cows' milk samples. Each milk sample was named according to the code of the cow from which each sample was collected (Table 4.1).

The milk samples were collected from individual cows during p.m. machine milking using on-farm milk collection bottles. A 50 ml milk sample was then transferred to plastic sterilized cups, and transported to the laboratory in ice-cooled containers. The milk samples were stored at 4 °C for a maximum of 12 h prior to analysis (Figure 4.1).

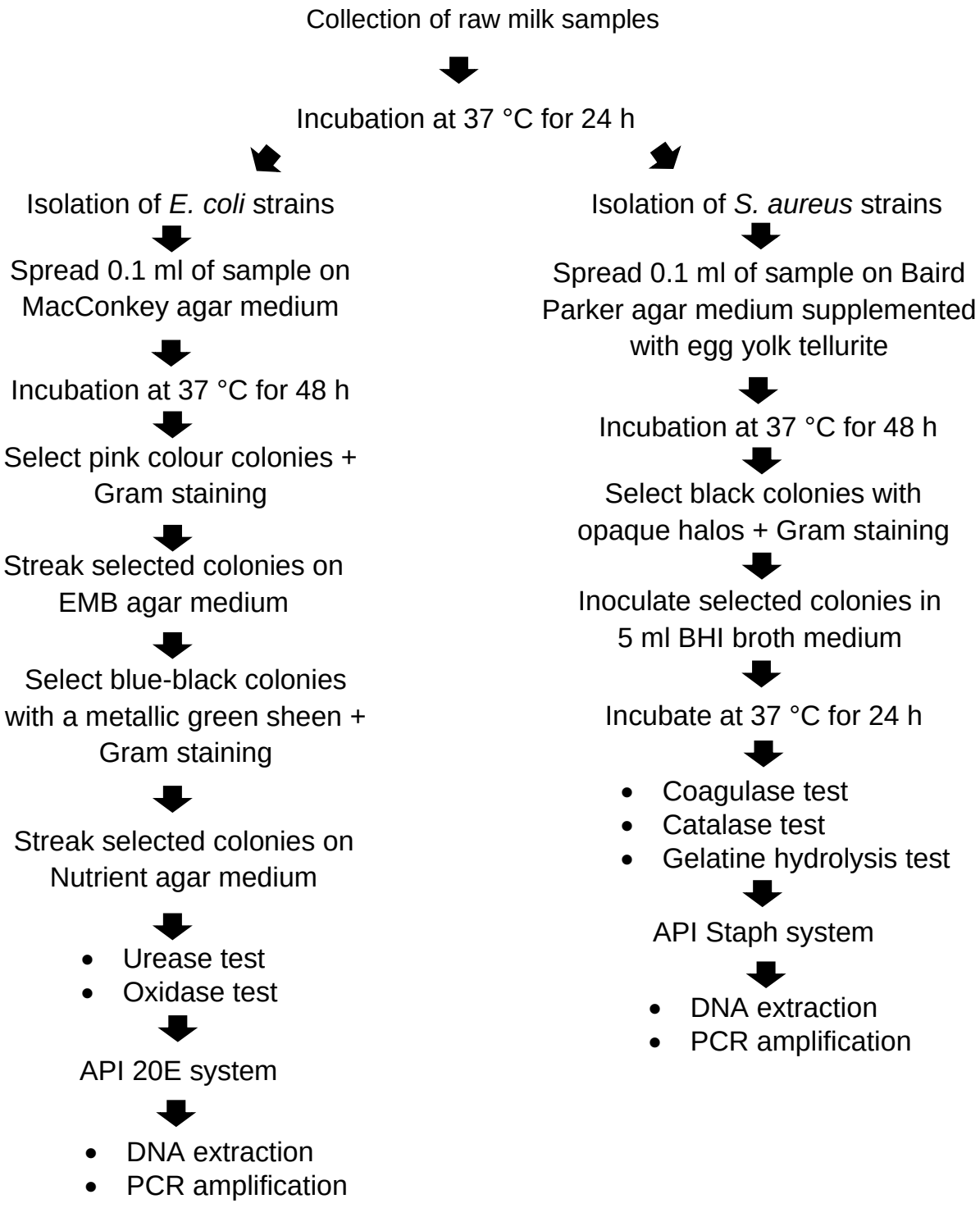

Figure 4.1. Flow diagram of the used technique for isolation of *E. coli* and *S. aureus* strains.

Table 4.1. The codes of the 100 collected raw cows' milk samples.

No.	Sample code*	No.	Sample code	No.	Sample code
1	3394	35	3559	69	4227
2	3198	36	3531	70	4024
3	2107	37	3874	71	3534
4	3437	38	3094	72	3605
5	3903	39	3708	73	2958
6	2706	40	2093	74	2712
7	3390	41	3951	75	3936
8	2792	42	2830	76	3471
9	3329	43	3507	77	102
10	3325	44	9365	78	3655
11	2861	45	3585	79	2887
12	3388	46	2960	80	4274
13	3991	47	2669	81	2840
14	3903	48	24	82	1
15	221	49	2890	83	2868
16	211	50	2954	84	4068
17	3412	51	3135	85	3379
18	3940	52	3977	86	324
19	5	53	3908	87	3939
20	3396	54	3497	88	3971
21	3132	55	4291	89	3983
22	2949	56	2790	90	4139
23	2942	57	3720	91	4304
24	3403	58	3516	92	4039
25	3317	59	23	93	3823
26	3871	60	3723	94	3308
27	3699	61	3301	95	3942
28	3629	62	4206	96	4388
29	2625	63	4001	97	2254
30	2606	64	3451	98	4183
31	3885	65	4002	99	4365
32	26	66	3783	100	3882
33	3391	67	3737		
34	2889	68	3666		

*Represent the code of the cow in HAU dairy unit which milk sample collected from.

4.2.3 Methods

4.2.3.1 Isolation of presumptive *E. coli* strains

4.2.3.1.1 Isolation of *E. coli* from collected milk samples

The previously collected raw milk samples were processed according to method EN ISO 6888 1-2 1999 in duplicate with some modifications. A volume of 0.1 ml of enrichment raw milk sample was spread on MacConkey agar medium (LABM, UK) in duplicate, and incubated at 37 °C for 48 h. Pink colonies were streaked on Eosin Methylene Blue (EMB) Agar (LABM, UK) (Ombarak *et al.*, 2016). Three to five presumptive colonies (blue-black with a metallic green sheen) from each EMB plate were picked, and then streaked on Nutrient Agar (NA) plates and incubated at 37 °C for 24 h for further testing (Figure 4.1). Gram staining was performed in each step as described in section 3.3.5. *E. coli* NCIMB 10243 was used as a reference strain (Table 3.2).

4.2.3.1.2 Oxidase test

The oxidase test was performed according to the method of the Health Protection Agency (NHS, 2019). A bacterial sample was scraped from the previously streaked NA plate (section 4.2.3.1.1) with a disposable loop and smeared onto filter paper. Around 2 or 3 drops of Kovac's oxidase reagent (Fisher Scientific, UK) were then added onto the smeared bacterial sample. After approximately 20 sec any colour change was observed and results recorded. *E. coli* NCIMB 10243 (Table 3.2) was used as a negative control and *Pseudomonas aeruginosa* (obtained from HAU culture collection) was used as a positive control.

4.2.3.1.3 Urease activity test

Urease activity was examined according to the method of the Health Protection Agency (NHS, 2015). A single colony from the previously NA plate (section 4.2.3.1.1) was used to inoculate 10 ml of Urea broth medium (Fisher Scientific, UK), then tubes were incubated at 30 °C for 6 h. *E. coli* NCIMB 10243 was used as a negative control (no colour change) and *Yersinia enterocolitica* DSM 11067 (Table 3.2) was used as a positive control (change to pink colour).

4.2.3.2 Isolation of *S. aureus* presumptive strains

4.2.3.2.1 Isolation of *S. aureus* from collected milk samples

The collected raw milk samples were processed according to method EN ISO 6888 1-2 1999 in duplicate with some modifications. A volume of 0.1 ml of enrichment raw milk sample was then spread on Baird-Parker agar medium with 5.0 % egg yolk tellurite (LABM, UK). Based on colony distinct morphological characteristics, three to five black colonies with opaque halos were picked, and then streaked on Nutrient Agar plates and incubated at 37 °C for 24 h for further testing (Figure 4.1). Gram staining was performed in each step as described in section 3.3.5. *S. aureus* NCTC 13552 was used as a reference strain (Table 3.2).

4.2.3.2.2 Coagulase activity test

Coagulase activity was carried out according to manufacturer's instructions as follows: A single colony from the previously prepared NA plate (section 4.2.3.2.1) was inoculated into 5 ml Brain Heart Infusion (BHI) broth medium (LABM, UK) and incubated at 37 °C for 24 h. Later, 0.5 ml of the incubated BHI broth was transferred aseptically to a sterile tube containing 0.5 ml of reconstituted rabbit plasma (Fisher Scientific, UK). The tubes were then incubated at 37 °C for up to 6 h to assess the coagulation activity. Additional incubation for another 12 h was done to confirm the negative reactions. *S. aureus* NCTC 13552 was used as a positive control and *S. epidermidis* DSM 28319 was used as a negative control (Table 3.2).

4.2.3.2.3 Catalase activity test

The catalase activity was performed as described by Prescott (2002). Three to five colonies from the previously prepared NA plate (section 4.2.3.2.1) were placed on a glass slide, and then was mixed in a 1 to 2 drops of 3 % hydrogen peroxide. *S. aureus* NCTC 13552 was used as a positive control (Table 3.2).

4.2.3.2.4 Gelatine hydrolysis test

The gelatine hydrolysis test was performed as described by Prescott (2002). A single colony from the previously prepared NA plate (section 4.2.3.2.1) was stabbed into a NA slant with 0.3 % bacteriological gelatine (Fisher Scientific, UK). The tube was then incubated at 30 °C for up to 7 days, and gelatine liquefaction was checked daily. As gelatine liquefies at temperature above 28 °C, the tubes were refrigerated for 30 minutes at 4 °C to confirm that liquefaction was due to gelatinase activity. *S. aureus* NCTC 13552 was used as a positive control and *S. epidermidis* DSM 28319 was used as a negative control (Table 3.2).

4.2.3.3 Biochemical examination

The API Identification system (BioMérieux, France) was used as per manufacturer's instructions. Presumptive positive isolates of *E. coli* and *S. aureus* were evaluated with the API 20 E and API Staph, respectively. The Apiweb™ (BioMérieux, Inc.) database (https://apiweb.biomerieux.com) was consulted and the results was assigned to each bacterial isolate tested. *E. coli* NCIMB 10243 and *S. aureus* NCTC 13552 were used as a reference strains.

A classification of ‘‘high probability’’ was assigned when the percentage identification accuracy (% ID), was greater than or equal to 80 and T-index more than 0.75, while the results were classified as ‘‘low probability’’ when the % ID was below 80. Finally, the positive isolates were stored in microorganism preservation beads (TSC Ltd., UK) at -80 °C until further use.

4.2.3.4 DNA extraction from bacterial cells

Approximately 10 colonies were obtained and suspended in 700 µL 5 % chelex solution (Yang *et al.*, 2008). The mixture was then heated at 95 °C for 20 min (with agitation each 5 min). After centrifugation at 10,000 rpm for 10 min, supernatants were transferred to a new 1.5 ml collection tube. All the DNA templates were stored at -20 °C until use with PCR amplification. DNA concentration and purity were evaluated by optical density using a NanoDrop ND-2000 spectrophotometer (Thermo-scientific, UK) using 260 – 280 nm wave length.

4.2.3.5 Confirmation of *E. coli* and *S. aureus* by PCR amplification

E. coli and *S. aureus* isolates from section 4.2.3.4.1 were identified using a multiplex polymerase chain reaction (PCR). PCR reaction mixtures were performed in a total volume of 25 µl, containing 12.5 µl of 2× dream Taq red master-mix, 0.5 µl of each forward and reverse primer (Eurofins, UK), 3 µl of DNA template and 8.5 µl nuclease free water. Finally, 2.5 µl of DNA template was added to each reaction tube.

E. coli isolates were confirmed using PCR as described by Carneiro *et al*. (2006) using selected primers as described in Table 4.2. Reactions were performed using a gradient PCR on the 96-well thermal cycler (T100, BIO-RAD, USA). For *eae*, *tir* and *esp* subtypes the following cycles were used 30× (94 °C for 30 sec, 52 °C for 30 sec and 72 °C for 90 sec). For *bfp* A, the following cycles were used: 29× (94 °C for 30 sec, 56 °C for I min, 72 °C for 2 min).

Table 4.2. Primers used to identify *E. coli* presumptive isolates.

Primer	Primer sequence (5´- 3´)	Amplicon size (bp)	Reference
eae β	TACTGAGATTAAGGCTGATAA TGTATGTCGCACTCTGATT	452	(China *et al.*, 1999)
eae ɣ	TACTGAGATTAAGGCTGATAA AGGAAGAGGGTTTTGTGTT	778	(China *et al.*, 1999)
tir α	CRCCKCCAYTACCTTCACA CGCTAACCTCCAAACCATT	342	(China *et al.*, 1999)
tir β	CRCCKCCAYTACCTTCACA GATTTTTCCCTCGCCACTA	560	(China *et al.*, 1999)
bfp A	AATGGTGCTTGCGCTTGCTGC GCCGCTTTATCCAACCTGGTA	326	(Carneiro *et al.*, 2006)
esp B α	GCCGTTTTTGAGAGCCA TCCCCAGGACAGATGAGAT	188	(Carneiro *et al.*, 2006)
esp B β	GCCGTTTTTGAGAGCCA CTTTCCGTTGCCTTAGT	233	(China *et al.*, 1999)
esp A α	TGAGGCATCTAARGMGTC GCTGGCTATTATTGACCG	269	(China *et al.*, 1999)
esp A β	TGAGGCATCTAARGMGTC TGCCTTTCTTATTCTTGTCA	101	(China *et al.*, 1999)

S. aureus isolates were confirmed using PCR as described by Brakstad *et al*. (1992) using the thermonuclease gene (*nuc*, *S. aureus* specific) (Table 4.3). After optimisation of PCR, amplification was performed using a gradient PCR on the 96-well thermal cycler (T100, BIO-RAD, USA) with initial denaturation step for 5 min at 94 ºC, gradient annealing step 35 cycles of 63 ºC for 30 sec, and a final extension at 72 ºC for 1 min. All PCR products were resolved by agarose gel electrophoresis and visualised on a UV transilluminator (212 PRO, Gel-Logic, USA).

Table 4.3. Primer used to identify *S. aureus* presumptive isolates.

Primer	Primer sequence (5´- 3´)	Amplicon size (bp)	Reference
nuc	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	279	(Brakstad, Aasbakk and Maeland, 1992)

4.3 Results & Discussion

4.3.1 *E. coli* screening in collected raw milk samples

In total, 48 *E. coli* presumptive colonies were isolated out of 100 raw milk samples (Table 4.4). Blue-black colonies with a metallic green sheen on EMB agar were picked and then characterized by colony morphology, Gram staining and biochemical examination. The biochemical identification using API 20 E showed that 23 isolates (48 %) had a high identification probability for *E. coli*, while 10 isolates (21 %) had low *E. coli* identification probability. On the other hand, two, five and four isolates showed high ID % for *Escherichia hermannii, Yersinia enterocolitica* and *Klebsiella pneumonia,* respectively.

Table 4.4. Phenotypic identification of *E. coli* presumptive isolates.

Species	Gram Stain	Urease Activity	Oxidase Activity	API 20 E	
				High probability (% ID ≥ 80)	Low probability (% ID < 80)
E. coli NCIMB 10243	-	-	-	+	-
E. coli	-	-	-	23*	10
E. hermannii	-	-	-	2	1
Y. enterocolitica	-	+	-	5	2
K. pneumonia	-	+	-	4	1

(-) Means negative result for the performed test
(+) Means Positive result for the performed test
*Number of the isolates

Similar to the achieved results*, Enterobacteriaceae* including *Escherichia coli*, *Klebsiella pneumonia* and *Yersinia enterocolitica* have been detected frequently in milk-borne diseases (Jamali *et al.*, 2015; Bonardi *et al.*, 2018). Ntuli, Njage and Buys (2016) isolated 697 *Enterobacteriaceae* isolates from 154 raw milk samples in South Africa which have been identified to 17.4 % *E. coli*, 2.3 % *Klebsiella pneumonia* and 1.7 % *Yersinia enterocolitica.* Moreover, Soomro *et al.* (2002) reported that 57 raw milk samples in Pakistan were positive for *E. coli* using culture and biochemical identification. These results are also in accordance with Uyanik *et al.* (2022) who isolated 41 *E. coli* and 6 *K. pneumonia* isolates by culture method out of 150 collected raw milk samples from the Black Sea region in Turkey.`

Enteropathogenic *E. coli* (EPEC) and Shiga toxin-producing *E. coli* (STEC) are from *E. coli* pathogenic groups that causing intestinal diseases (Malik *et al.*, 2017; Rios *et al.*, 2019). As a considerable amount of literature has been published on prevalence of *E. coli* (EPEC) in samples of cow's milk or cheese manufactured from raw cow's milk (Carneiro *et al.*, 2006; Aidar-Ugrinovich *et al.*, 2007; Beraldo *et al.*, 2014; Álvarez-Suárez *et al.*, 2016; Rios *et al.*, 2019), and the variants of *eae*, *bfp tir*, *esp* A and *esp* B genes were examined for EPEC identification (Sanches *et al.*, 2017; Haymaker *et al.*, 2019).

Of the strains that showed high identification probability for *E. coli* (23 isolates), sixteen isolates were positive for most *E. coli* genes (Table 4.5). The *bfp* A and *eae* (β and ɣ) genes were detected in 4 (22 %) and 13 (72 %), respectively of the *E. coli* isolates. In addition, *tir* (α and β), *esp* B (α and β) and *esp* A (α and β) genes were detected in 15 (83 %) of the *E. coli* isolates. This is in accordance with Ribeiro Júnior *et al.* (2018) who detected *bfp* A gene in 70 % (14) of *E. coli* isolates from raw milk samples in Brazil.

Table 4.5. Molecular identification of *E. coli* isolates by PCR.

Strain code	Identified PCR primers								
	eae		*tir*		*bfp* A	*esp* B		*esp* A	
	β	ɣ	α	β		α	β	α	β
3760	+	+	+	+	-	+	+	+	+
2254	+	+	+	+	-	+	+	+	+
3748	-	-	+	+	+	+	+	+	+
3394	+	+	+	+	-	+	+	+	+
3421	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-
3198	-	-	+	+	+	+	+	+	+
3709	+	+	+	+	-	+	+	+	+
102	+	+	+	+	-	+	+	+	+
4024	+	+	+	+	-	+	+	+	+
3977	+	+	+	+	-	+	+	+	+
4001	-	-	-	-	-	-	-	-	-
4068	-	-	-	-	-	-	-	-	-
4291	+	+	+	+	-	+	+	+	+
3301	+	+	+	+	+	+	+	+	+
324	-	-	-	-	-	-	-	-	-
4022	+	+	+	+	-	+	+	+	+
4139	-	-	-	-	-	-	-	-	-
212	+	+	+	+	-	+	+	+	+
3761	+	+	+	+	-	+	+	+	+
3723	-	-	-	-	-	-	-	-	-
2259	+	+	+	+	-	+	+	+	+
2958	-	-	+	+	+	+	+	+	+

(-) Means absence of the target gene

(+) Means presence of the target gene

EPEC can be classified as typical (tEPEC) and atypical (aEPEC), where both produce attaching and effacing lesions. The responsible genes for this lesion are clustered on the chromosome forming pathogenesis island called Locus for Enterocyte Effacement (LEE). The LEE carries genes coding for: intimin (*eae*), a type III secretion protein (*esp* A, *esp* B) and the translocated intimin receptor (*tir*). Furthermore, the bundle-forming pilus (*bfp*) of EPEC, which is an established virulence factor encoded on the EPEC adherence factor (EAF) plasmid, is only found in tEPEC (Carneiro *et al.*, 2006; Hernandes *et al.*, 2009; Sanches *et al.*, 2017).

Accordingly, these results showed variability between typical and atypical EPEC, where 12 *E. coli* isolates (Table 4.5) were aEPEC, three *E. coli* isolates were tEPEC and one *E. coli* isolate was positive for both *eae* and *bfp* genes. These results may therefore give a better understanding of the origin of *E. coli*

contamination, as the only reservoir of tEPEC is humans, while aEPEC can be found in both animals and humans (Trabulsi, Keller and Gomes, 2002; Carneiro *et al.*, 2006).

4.3.2 *S. aureus* screening in collected raw milk samples

Raw milk screening resulted in a total of 52 presumptive *Staphylococcus* spp. isolates out of 100 samples (Table 4.6). Colonies with an opaque halo were picked; and then characterized by morphology, Gram stain and biochemical examination. The biochemical identification using API Staph showed that 24 isolates (46 %) had a high identification probability for *S. aureus*, while six isolates (12 %) had low *S. aureus* identification probability. Three *S. hyicus* strains were also isolated with high ID %.

Table 4.6. Phenotypic identification of *S. aureus* presumptive isolates.

Species	Gram Stain	Gelatin hydrolysis	Catalase Activity	Coagulase activity	API Staph	
					High probability (% ID ≥ 80)	Low probability (% ID < 80)
S. aureus NCTC 13552	+	+	+	+	+	-
S. aureus	+	+	+	+	24*	6
S. epidermidis	+	-	+	-	3	1
S. hyicus	+	-	+	+	3	1
S. haemolyticus	+	-	+	-	5	5
S. warnerri	+	-	+	-	1	1
S. xylosus	+	-	+	-	1	1

(-) Means negative result for the performed test
(+) Means Positive result for the performed test
*Number of the isolates

Staphylococcus aureus is widely known as the main cause of foodborne illnesses in humans resulting from consumption of raw milk or their products (Omwenga *et al.*, 2019). In parallel with achieved results, S. *aureus* has been reported to be the most prevalent Staphylococcal spp. by 12.9 % (484 isolates) after testing of 3760 samples of raw milk and its dairy products (Basanisi *et al.*, 2017). Similarly, Thaker, Brahmbhatt and Nayak (2013) have isolated 10 *S.*

aureus isolates out of 100 raw milk samples in India by culturing and biochemical examination.

On the other hand, existence of *S. hyicus* in raw milk is supported by de Freitas Guimarães *et al.* (2013) who collected 1203 raw cow's milk samples in order to isolate coagulase-positive staphylococci (CPS) and coagulase-negative staphylococci (CNS); the results showed that 91 (68 %) and 17 (13 %) of CPS isolates were *S. aureus* and *S. hyicus*, respectively.

The isolates that showed negative result for coagulase test were identified by API Staph. The achieved results showed high ID % for *S. epidermidis* (three isolates), *S. haemolyticus* (five isolates), *S. warnerii* (one isolate) and *S. xylosus* (one isolate). This is linked to previous studies that reported the high prevalence of *S. epidermidis* and *S. haemolyticus* between coagulase-negative staphylococci (CNS) in raw milk (de Freitas Guimarães *et al.*, 2013; Condas *et al.*, 2017). Corbeil *et al.* (2019) isolated five (7 %) *S. haemolyticus* and five (7 %) *S. epidermidis* out of 65 raw cow's milk samples in Canada.

In Spain, Alnakip *et al.* (2019) have reported that five (8.2 %) *S. aureus*, four (6.6 %) *S. epidermidis* and two (3.28 %) *S. haemolyticus* were isolated out of 100 raw cow's milk samples. Furthermore, one *S. warnerii* strain was isolated from collected raw milk samples; which is in line with Klibi *et al.* (2018) who have demonstrated that 12 % of the 68 staphylococci spp. isolated from 300 raw milk samples were identified as *S. warnerii*.

Of the strains that showed high identification probability for *S. aureus* (24 isolate), 20 isolates (3421, 2254, 2958, 3977, 3743, 3748, 2933, 3749, 3747, 4365, 3741, 3742, 324, 3983, 4304, 2790, 3720, 3516, 23 and 3723) were confirmed using PCR detection of the thermonuclease gene (*nuc*; *Staphylococcus aureus* specific gene). These data demonstrate the high accuracy of used classical culturing and biochemical examination, which is supported by Özdemir and Keyvan (2016) who identified 114 (78 %) out 146 CPS isolates using similar to the followed biochemical and molecular identification technique.

4.4 Conclusions

Enrichment of raw milk samples followed by isolation using the previously mentioned techniques showed a valid way for isolation of *E. coli* and *S. aureus* strains from raw milk. The demonstrated results revealed an existence of *E. coli* and *S. aureus* strains in raw cow's milk collected from HAU dairy unit.

As the objective of this work is isolation of *E. coli* and *S. aureus* strains, we cannot judge the microbiological quality of HAU cows' milk. However, these data revealed that most of the *E. coli* and *S. aureus* isolates were from animal origin with a limited number of human origin strains. This emphasises the need to monitor the sanitary condition of the plant and milking utensils, as well as milking personnel hygiene. Accordingly, the identified *E. coli* and *S. aureus* isolates by both biochemical and molecular examination is necessary to build a large culture collection for testing of bacteriophage host ranges (section 6.3.3).

Chapter Five: Determination of the Critical Physicochemical Conditions of Egyptian Raw Milk Cheeses During Small Scale Manufacture and Storage

5.1 Introduction

The most popular cheeses in Egypt, Karish, Domiati and Ras, are manufactured from raw cows' or buffalos' milk following traditional process as described in section 3.2. Previous studies have reported that there are several factors which could be directly or indirectly linked to the variation in milk chemical composition (section 2.6.3.1.1). Particularly, changes in cow breed (Bittante *et al.*, 2021), season of lactation (Parmar *et al.*, 2020), animal feed (Manzocchi *et al.*, 2021) and geographic origin (Guo *et al.*, 2021) can affect milk composition quantity and proteins/fats quality , and level of associated microbiota (Nalepa, Olszewska and Markiewicz, 2018).

Researchers observed that the recorded variations in milk composition were attributed to changes in the physiological performance of the mammary gland, that affects mainly: a) the protein and fat quantity in milk, b) proportions of individual caseins and lipid triglycerides, and c) enzymatic activities (Fox *et al.*, 2017b). As such, in Egypt Kabil *et al.* (2015) studied the chemical composition of 100 individual cow's milk samples from different regions during the four seasons. The authors found that in winter, the milk protein and fat content were 3.6 and 3.5 %, respectively, however in summer these percentages decreased to 3.1 for both protein and fat. Similary, in UK, Chen, Lewis and Grandison (2014) reported that pH value, protein, total casein and fat % increased significantly ($p \leq 0.05$) in

spring (March, April and May) compared to summer and autumn, after testing raw bulk milk (from 550 cows).

Cheese making is also an intricate process, involving many steps such as: milk clotting, whey separation, salting and ripening; some of which are essential for nearly all cheese varieties (Fox *et al.*, 2017c). Consequently, any changes in processing could lead to production of different types of cheese with varied physicochemical properties (CDC and Health Canada, 2015; Hickey *et al*., 2017). Accordingly, the variation in milk type, season or milking environment will subsequently affect the physicochemical conditions of manufactured cheese (Todaro, A. Adly and Omar, 2013). For example, variation mainly in milk composition and associated natural microbiota would lead to readjustment of amount of rennet required, amount of starter culture (if required), maturation time and ripening process (Fox *et al*., 2000; Al-Nabulsi *et al.*, 2020; Manzocchi *et al.*, 2021).

As the standard way to manufacture Karish, Domiati and Ras cheeses is from Egyptian cows' milk and under Egyptian environmental conditions; due to variation in region of study and the manufacture at small scale laboratory in the present study. Therefore, the objectives of this work (phase 2, section 3.1) are to;

i) evaluate, adapt and validate the existing protocols for Karish, Domiati and Ras manufacture under small scale laboratory conditions to be followed in testing the phage efficacy as biocontrol agent in raw milk Egyptian cheese (Phase 5, Chapter 8);

ii) identify the critical physicochemical conditions that the bacteriophage will be exposed to during assessment of phage biocontrol efficacy under *in vitro* evaluation (phase 3, Chapter 6).

5.2 Experimental Setup

5.2.1 Study design

This study was designed to validate Karish, Domiati and Ras manufacture protocols at small scale laboratory conditions in order to reach a final product with physicochemical properties close to the artisanal cheese. Three types of raw milk Egyptian cheeses (Karish, Domiati and Ras) were manufactured in duplicate in the HAU category two containment laboratory over a period of four months from April to August 2018. Karish, Domiati and Ras cheeses were manufactured as described in sections 3.2.1, 3.2.2 and 3.2.3, respectively.

5.2.2 Sampling & data collection

The Karish, Domiati and Ras cheeses were stored for 15, 90 and 180 days, respectively; the selected timed period was related to the standard shelf life for each cheese as described in section 2.2.2. Samples (50 g) were collected at different intervals as shown in Table 5.1 and placed in sterile plastic cups (250 ml), sealed (to prevent dehydration) and stored at 4 °C for pH, composition and water activity analysis. The sampling technique and pH measurement were carried out as described in section 3.2. After testing, samples were stored at -20 °C for any subsequent analysis. Samples were left at room temperature for approximately 30 – 45 min prior to testing. The texture profile of Karish, Domiati and Ras cheeses were analysed at the end of each cheese storage period.

Table 5.1. The interval stages for collection of Karish, Domiati and Ras cheese samples.

Cheese	Coagulation time		Cold storage or ripening time			
Karish	Milk	Curd*	Day** 1	Day 5	Day 9	Day 15
Domiati	Milk	Curd	Day 1	Day 14	Day 42	Day 90
Ras	Milk	Curd	Day 1	Day 30	Day 60	Day 180

*The stage of complete curdling after 24 h of starter addition for Karish or after 3 h and 45 min of rennet addition during Domiati and Ras manufacture, respectively.

**Day at which the cheese manufacture process was completed and kept for storage or ripening.

5.2.3 Methods

5.2.3.1 Determination of moisture content

The moisture percent in curd and cheese samples was determined according to International Dairy Federation (IDF) Standard (IDF, 1982) in duplicate. A subsample of curd or cheese was accurately weighed (~ 5 g) and oven dried at 105 °C for around 1 h, until constant weight was reached. Samples were cooled in a desiccator and re-weighed. Moisture content % was calculated as:

$$Moisture\ \% = \frac{(\text{Initial sample weight} - \text{Weight of dry sample})}{\text{Initial sample weight}} \times 100$$

5.2.3.2 Determination of fat content

The fat percentage in curd and cheese samples was measured by Gerber method (AOAC, 2003) in duplicate. Using a dry cheese beaker stopper (FG3321, Funke Gerber, Germany) a 3 g of sample was weighed. After that, in a cheese butyrometer (FG3230, Funke Gerber, Germany), 6 ml of distilled water at 15 – 21 °C was added, then 10 ml of sulphuric acid (LABTEK, UK) and 1 ml of isoamyl alcohol (LABTEK, UK) were added slowly. The cheese beaker stopper containing tested sample was inserted into the butyrometer and closed securely. The butyrometer was shaken well until all traces of curd disappeared. Butyrometers were centrifuged (Funke Gerber, Germany) for 4 min at 60 – 63 °C. After centrifugation, butyrometers were left for 5 min to equilibrate the fat column. The fat percentage was then measured directly from the reading scale.

5.2.3.3 Determination of protein content

Percentage of protein in curd and cheese samples was determined by measuring the total nitrogen using Kjeldahl method (AOAC, 2003) in duplicate. A 3 g sample was firstly transferred into Kjeldahl digestion tube (Foss Tecator Digestor Unit, Denmark) for digestion using 5 ml sulphuric acid. Secondly, the digested sample was distilled in manual tecator (Kjeltec 8400, Foss, Denmark).

The protein % was calculated (Fox, Guinee and Cogan, 2000) as:

$$Protein\ \% = Total\ nitrogen\ \% \times 6.38$$

5.2.3.4 Determination of salt concentration

Salt concentration was determined in cheese samples only for Karish and Ras (Table 5.1), however, for Domiati cheese, salt was analysed in milk, curd and cheese samples as the salt is added to milk at the beginning of Domiati cheese manufacture (section 3.2.2). Salt content was measured in duplicate using a titrimetric method known as the Mohr method (Öksüz *et al.*, 2004). The previously oven dried sample (section 5.2.3.1) was accurately weighed (4 g) into pre-weighed porcelain cups and heated overnight at 550 °C in a carbolite muffle furnace (AAF 1100, Hope Valley, England). Samples were then cooled in a desiccator and re-weighed. After that, 0.5 ml of potassium chromate (K_2CrO_4, 0.5 N) and 25 ml of distilled water were mixed with previously ashed sample. The mixture was then titrated using silver nitrate ($AgNO_3$, 0.05 N) until a brown colour developed. A blank sample using only distilled water was also performed.

The salt % was calculated as:

$$Salt\ \% = \left(\frac{\text{Titrated vol. of silver nitrate} \times 0.00292}{\text{Original sample weight (g)}}\right) \times 100$$

5.2.3.5 Determination of water activity

The water activity (A_w) in milk, curd and cheese samples was measured in duplicate using water activity meter (Labmaster Neo AW, UK). Samples were filled in a disposable sample cup, ensuring the homogeneity of curd or cheese samples and complete covering of the bottom of the cup. The extra hard part (rind) in the outer 2 cm of Ras cheese sample was trimmed to ensure accuracy of results.

5.2.3.6 Texture profile analysis of Karish, Domiati and Ras cheeses

Texture profile analysis (TPA) of cheese samples was performed in duplicate as described by Sameh (2016) for Karish and Domiati cheese, and by Awad (2006) for Ras cheese. TPA was performed using twin column texture analyser (TA. HD *plus C* Texture Analyser, Stable Micro Systems, UK) with collecting 10 individual readings per replicate. A TA-16A Perspex cone was used as the probe with a two-bite penetration of 10 mm at 1 mm/sec (Figure 5.1). Textural characteristics were calculated using Texture Analyser Computer

Software (Stable micro systems TA HD Plus, UK). Karish and Domiati samples presented to the instrument were 40 mm in diameter and 30 mm in height. For Ras cheese, samples were cut into cheese cubes (20 × 20 × 20 mm). All samples were kept in plastic cups and sealed (to prevent dehydration).

The following textural parameters were determined: hardness (the maximum force achieved at the first bite); cohesiveness (the ability of sample to withstand the second deformation relative to its resistance under the first deformation); springiness (the length of 2^{nd} compression/ the length of the 1^{st} compression); and chewiness (the energy required to chew a food product to a state where it is ready for swallowing). The parameters of texture profile analysis are illustrated in Figure 5.2.

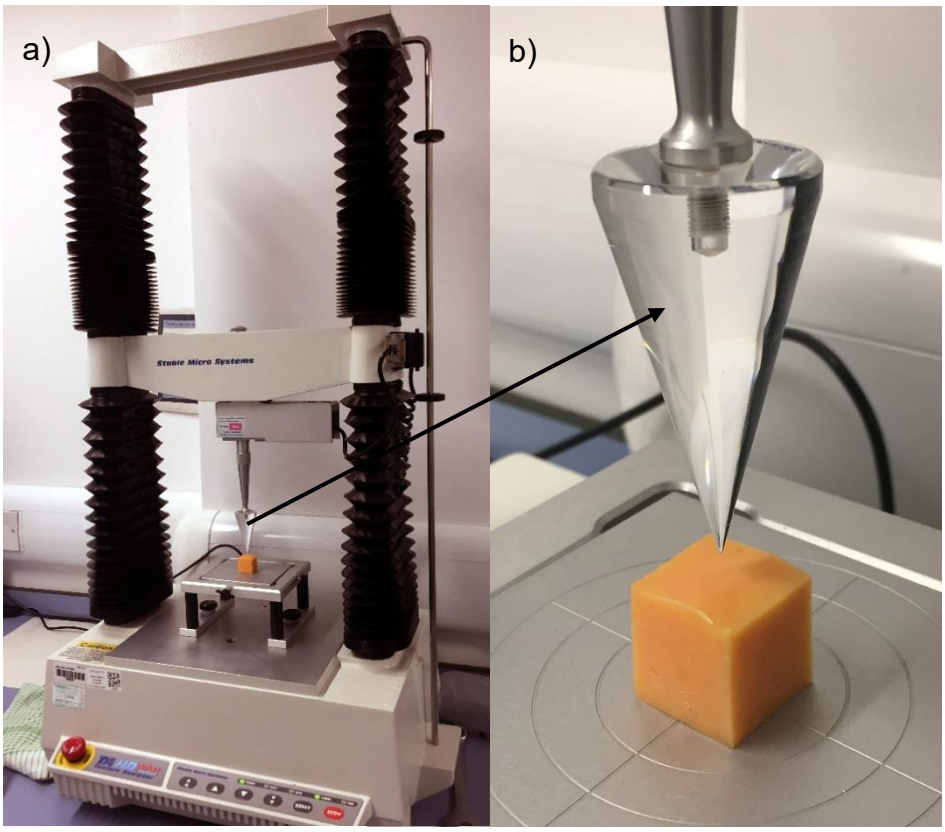

Figure 5.1. Typical TPA of Ras cheese using twin column texture analyser (a) and TA-16A Perspex cone probe (b).

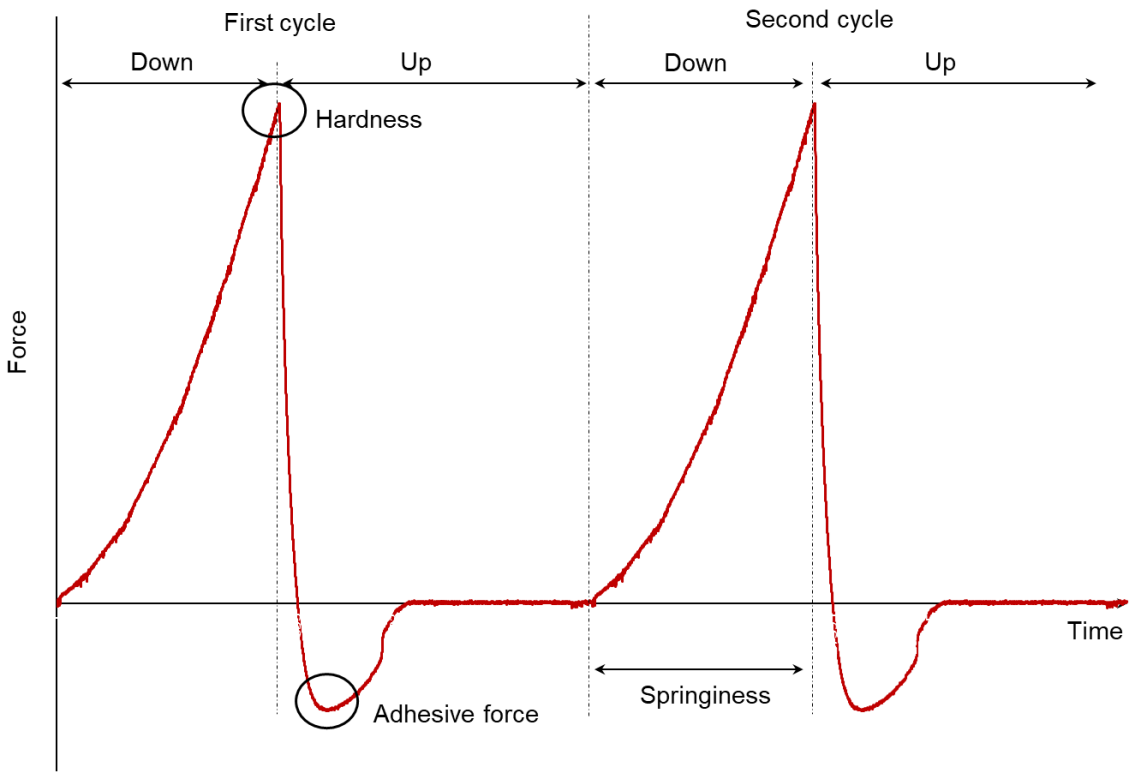

Figure 5.2. Example of obtained instrumental texture profile curve with two-bite penetration for each individual reading with illustration of the specific area for calculation of hardness, adhesive force and springiness.

5.2.4 Statistical analysis

Results are presented as means ± standard deviation (n = 4). A general design analysis of variance (ANOVA) was carried out to determine any significant differences ($p \leq 0.05$) among the treatments and Duncan's multiple range test ($p \leq 0.05$) was applied to compare the average values obtained between control and treatment using GenStat software (GenStat V 18.1. 17005, England, UK).

5.3 Results & Discussion

5.3.1 Karish cheese physicochemical conditions during small scale manufacture and storage

The physicochemical conditions of Karish cheese during coagulation time (milk and curd) and cold storage (1, 5, 9 and 15 days) are illustrated in Figure 5.3. The achieved results showed a significant ($p \leq 0.05$) reduction in pH value from 6.7 in milk to 4.6 in curd (Figure 5.3a); and pH reduction ($p = 0.203$) was continued to reach 4.3 after 15 days. Moreover, the moisture content was 79.2 % in curd and decreased significantly ($p = 0.008$) to 72.1 % at day 1 (Figure 5.3b). However, moisture content was stable during cold storage, as it decreased to 71.2 % after 15 days of storage. Interestingly, obtained skimmed milk had 0.5 and 3.1 % fat and protein, respectively, that showed the high efficiency of the followed milk fat separation and standardization technique. In addition, Figure 5.4b shows that there was inverse relationship between moisture content and protein and fat content.

Furthermore, the salt content in Karish was analysed after 1, 5, 9 and 15 days of storage (Figure 5.3c). The obtained results showed that salt content recorded 0.5 % at day 1, and decreased gradually to 0.4 % after 15 days of storage. The recorded Karish pH reduction during storage would enhance whey separation, which subsequently could justify the salt reduction in cheese ample at the end of storage. The high moisture and low salt content of Karish resulted in high recorded water activity (between 0.971 and 0.968) in milk and cheese until the end of storage period. (Figure 5.3d).

Overall, the achieved Karish cheese physicochemical conditions were close to the reported results as described in section 2.2.3. The decline in the pH values indicates the good fermentation activity of the added starter culture during 24 h of incubation at 37 °C. These results are in consistent with Sameh (2016) and Allam *et al.* (2017) findings, who studied the chemical composition of artisan-produced Karish collected from local markets in different regions in Egypt. The average pH values were between 4.2 – 4.7 at day 1 and between 3.9 – 4.4 after 15 days of storage. In addition, Ahmed *et al.* (2005) stated that Karish pH values ranged between 4.5 and 4.4 at day 1 and 15 day of storage, respectively; where Karish cheese was made using a starter culture of *Lb. bulgaricus* and *St. thermophilus*. The authors added that Karish moisture, protein and fat percentages were 71.4 ± 0.4, 17.4 ± 0.3 and 0.4 ± 0.2 %, respectively, when manufactured from pasteurized milk (fat = 0.1 %) and using *Lb. bulgaricus* and *S. thermophilus* as starter culture. These results are similar to achieved results, however, the observed variation in cheese fat % is related to the lower milk fat content used in their study.

Similarly, many researchers reported that the moisture content of tested Karish was from 66.0 to 74.0 % with an average of 71.0 % at day 1 (fresh) and 68.0 % after 15 days of storage (Hussein and Shalaby, 2014; Allam *et al.*, 2017). However, the observed stability in moisture content during storage could be attributed to the used vacuum packaging in cheese manufacture, as it decreased whey release from the cheese during storage. Little attention in literature was been paid for testing the Karish cheese water activity, that may be related to the higher water content and low salt (0.5 ± 0.3 %) (Ahmed *et al.*, 2005; Allam *et al.*, 2017), so the high Karish cheese A_w may be expected.

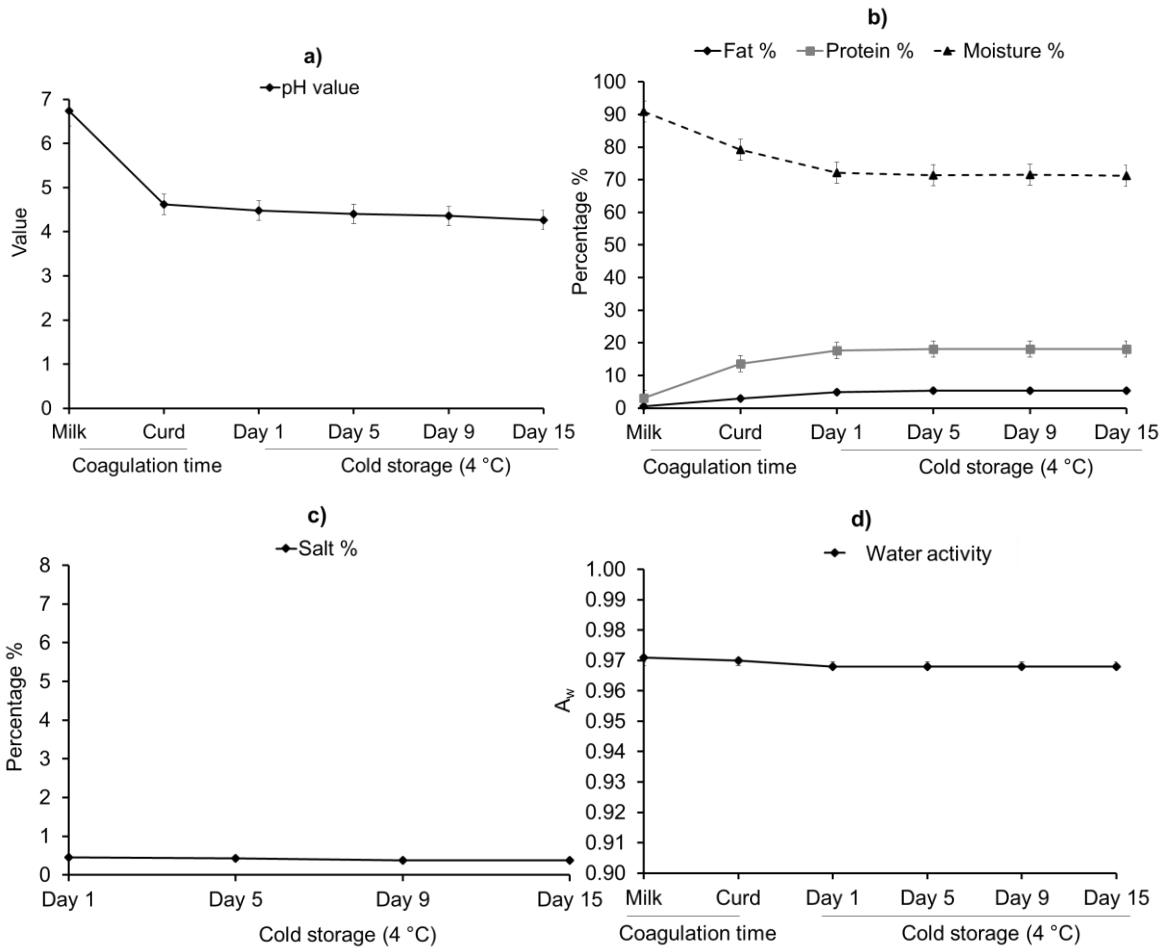

Figure 5.3. Changes in Karish cheese a) pH value; b) fat, protein and moisture %; c) salt concentration %; and d) water activity (A_w) during coagulation and cold storage at 4 °C. Data reported are means ± standard deviations of two independent trials.

5.3.2 Domiati cheese physicochemical conditions during small scale manufacture and ripening

The physicochemical conditions of Domiati cheese during coagulation (milk and curd) and ripening (1, 14, 42 and 90 days) are illustrated in Figure 5.4. The achieved results showed that milk pH value was 6.7 that decreased (p= 0.481) gradually to 6.3 at the end of 90 days at 4 °C (Figure 5.4a). Moreover, the moisture content decreased ($p \leq 0.05$) from 88.0 % in raw whole milk to 71.1 % in curd and then to 64.1 % at day 1, and continuous reduction (p= 0.203) to 59.7 % was recorded up to 90 days of Domiati ripening in brine solution. Additionally, protein and fat percentages increased ($p \leq 0.05$) to 18.2 and 16.5 %, respectively at day 1; and linear increase of Domiati protein and fat content to 24.9 and 21.4 %, respectively were recorded after 90 days (Figure 5.4b).

Of note, there was a clear correlation between the salt content (Figure 5.4d) and water activity (Figure 5.4c). Salt concentration was decreased significantly ($p \leq 0.05$) from 7.9 % in milk to 4.2 % at day 1, whereas salt % increased gradually again to 7.0 % after 90 days of ripening. This recorded high salt content in Domiati led to lowering the water activity (A_w) to 0.904 and increased ($p \leq 0.05$) to 0.917 at day 1, where A_w was decreased again to 0.901 after 90 days.

These results showed that adapted manufacture protocol include mainly renneting, milk salting and ripening conditions in brine solution were close to the artisanal Domiati cheese. According to, Abd El-Salam *et al*. (1993) the pH of traditional Domiati cheese manufactured from cows' or buffalos' milk without addition of starter culture is ranged between 6.0 and 6.5. In addition, pH values of Domiati cheese with added starter culture ranged between 6.5 in fresh to 5.3 after 90 days of ripening (Ayad, 2009). As described in literature (section 2.2.3), the addition of starter culture in Domiati cheese manufacture is optional; it can be used to improve safety or flavour of Domiati (Ayad, 2009; Awad, Ahmed and El Soda, 2010). However, the inoculated starter culture and natural microbiota did not decrease the pH of milk until the end of ripening, that implies their inability to grow at high salt content.

On the other hand, Ayad (2009) screened the physicochemical properties of Domiati cheese during manufacture from pasteurized whole milk with 5.0 % salt and 2.0 % starter culture (*L. lactis* and *L. cremoris*) up to 3 months of ripening at 10 °C. Ayad stated that Domiati pH was ranged between 6.5 at day 1 to 5.3 after 3 months, where the recorded low pH values is attributed to the high dose of starter. Furthermore, Ayad recorded a 60.2 and 22.1 % for moisture and fat, respectively. Although it was observed an increase in salt content during ripening, Ayad found that Domiati salt content decreased to 3.4 % after 3 months, this variation could be mainly linked to the salt concentration in the used brine solution, that was not indicated in Ayad study.

In accordance to the obtained results, Atallah, El-Deeb and Mohamed (2021) found that Domiati cheese had 61.1 ± 0.5, 12.5 ± 0.4 and 20.0 ± 0.5 % of moisture, protein and fat, respectively after 45 days of ripening. Additionally, they recorded an increase in Domiati salt content from 4.0 to 4.8 % after 45 days of ripening in 4.0 % brine. Generally, Domiati moisture content is decreased during ripening, which is attributed to the loss of the moisture into the brine solution during the ripening period (Ismail *et al.*, 2011)**.** The achieved chemical composition results of Domiati cheese are in agreement with the typical composition of artisanal Egyptian Domiati cheese (El-Baradei, Delacroix-Buchet and Ogier, 2007; Awad, Ahmed and El Soda, 2010; Ibrahim and Awad, 2018), and are within the legal limit of Domiati in Egypt (Egyptian standard 1008-3/ 2005).

The recorded fluctuation in salt content is associated with the separation of salted whey and restorage into the brine solution (with 8.0 % salt). In this study, the salting process was performed to give approximately 8.0 % salt in Domiati cheese; this was selected to be a middle point between 5.0 and 15.0 % salt that could be found in Domiati (El-Baradei, Delacroix-Buchet and Ogier, 2007; Guinee and Sutherland, 2016). The research to date in Domiati has tended to focus on salt concentration rather than the reflection of salt on water activity. A relative to Domiati cheese, white-brined cheese (stored in 10.0 to 15.0 % salt brine solution) had water activity ranged between 0.92 to 0.87 (Al-Nabulsi *et al.*, 2020).

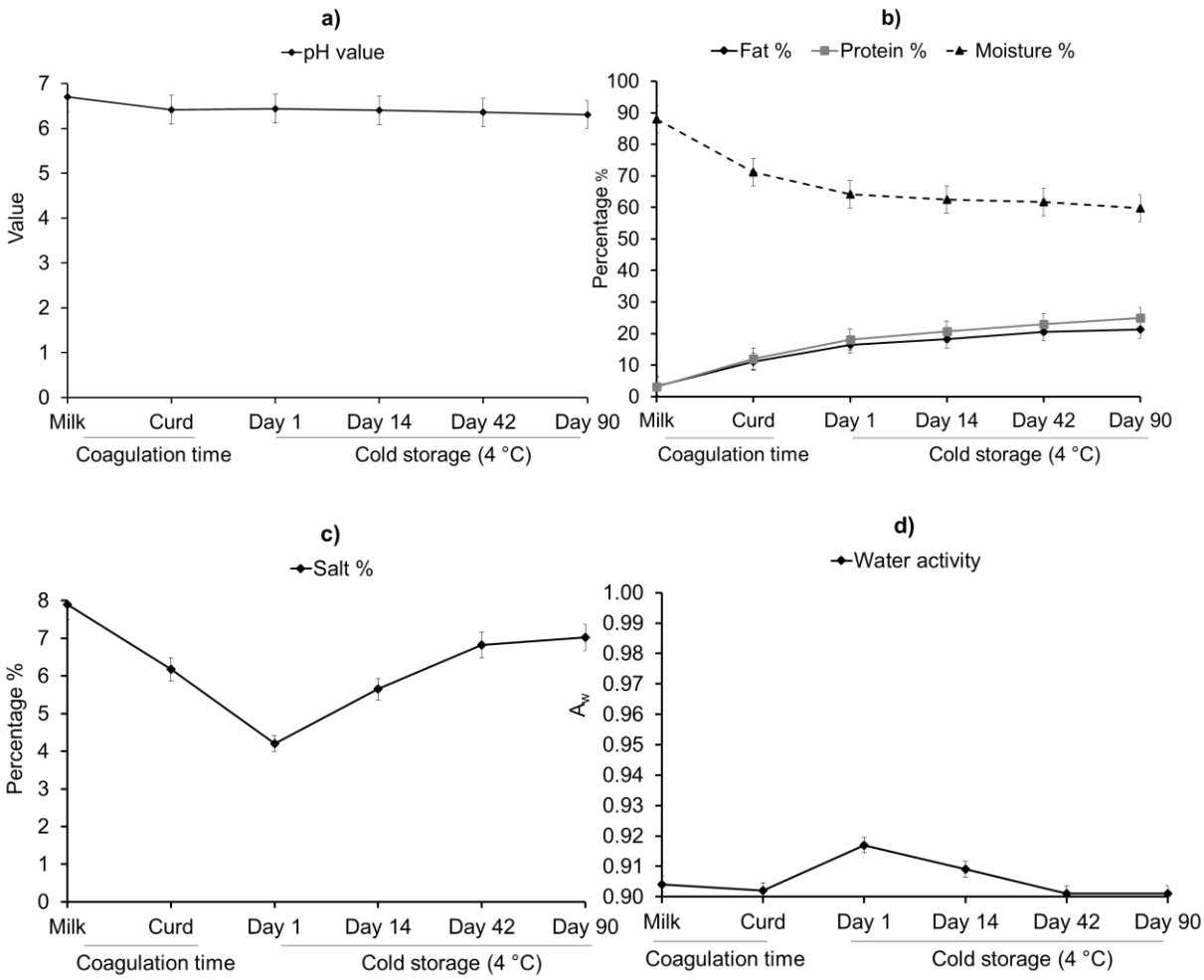

Figure 5.4. Changes in Domiati cheese a) pH value; b) fat, protein and moisture %; c) salt concentration %; and d) water activity (A_w) during coagulation and ripening at 4 °C. Data reported are means ± standard deviations of two independent trials.

5.3.3 Ras cheese physicochemical conditions during small scale manufacture and ripening

The physicochemical conditions of Ras cheese during coagulation time (milk and curd) and ripening time (1, 30, 60 and 180 days) are illustrated in Figure 5.5. The achieved results showed that pH value was decreased significantly ($p \leq 0.05$) to 5.9 in curd. Although pH reduction was continued to 5.4 after 30 days, the Ras pH values increased (p= 0.621) back gradually to 5.6 after 180 days of ripening (Figure 5.5a).

Figure 5.5b revealed that Ras moisture content showed a big decrease from 87.9 % in milk to 39.2 % at day 1 of cheese, which related to the separation of whey during curd cutting, scalding and cheese pressing. Moreover, the ripening process of Ras for 180 days at 9 to 12 °C and 85 % relative humidity (RH) led to a significant reduction (p= 0.009) in moisture to 30.1 % accompanied with a significant increase ($p \leq 0.05$) in fat and protein percentages from 27.1 and 22.8 % at day 1 to 34.0 and 26.7 % at the end of ripening, respectively.

In this study, the salting process was performed by dry salting of Ras cheese blocks during the first 12 days of ripening to achieve between 3.5 and 4.0 % salt. This technique was shown to be effective in increasing the salt % significantly ($p \leq 0.05$) from 0.5 at day 1 to 3.5 and 4.1 % after 90 and 180 days, respectively (Figure 5.5d). The recorded decrease in moisture content and increase in salt content was shown to be the reason of the achieved linearly ($p \leq 0.05$) reduction of water activity from 0.97 in milk to 0.92 after 180 days of ripening (Figure 5.5c).

The added starter culture and natural milk microbiota showed a good fermentation activity, that was also recorded with Karish cheese. However, the increase in pH at the end of ripening could be assigned to the formation of alkaline products such as amino acids, liberated from milk proteins by LAB proteolytic enzymes (Awad, 2006; Awad, Ahmed and Soda, 2007). These results are in concurrence with several studies, that investigated the physicochemical conditions of Ras cheese manufactured from Egyptian milk with or without using starter cultures (Hofi *et al.*, 1970; Ayad *et al.*, 2004; Awad, 2006; Youssef *et al.*, 2019).

Awad, Ahmed and Soda (2007) was the first study to investigate the use of starter culture in Ras cheese manufacture from pasteurized milk. They reported that Ras had 46.8 % moisture at day 1, which decreased to 36.1 and 35.2 % after 60 and 120 days of ripening, respectively. In addition, protein and salt content were 21.0 and 1.7 % respectively, at day 1, that increased gradually to 24.0 (protein) and 4.0 (salt) %. The authors added that the increase or decrease of pH values were more strain dependent, where during ripening of Ras, pH increased with cultures that showed high enzymatic activity, which subsequently resulted in a higher recorded free amino acids and free fatty acids; this observation is also supported by Abd-Elmonem *et al.* (2022). According to these data, it could be assumed that used starter and natural raw milk microbiota had a high proteolytic and lipolytic activity, because of the achieved increase in pH values.

Similarly, Abd-Elmonem *et al.* (2022) studied the use of *Lb. acidophilus*, *Lb. helveticus* and *Lb. casei* as starter culture (1.0 %) in Ras cheese manufacture. Abd-Elmonem *et al.* recorded that manufactured Ras had a 44.4 % moisture at day 1 that decreased gradually to 30.3 % after 90 days of ripening period at 13 ± 2 °C at 85 % RH. In addition, the salt content of Ras was 3.3 and 4.5 % after 1 and 90 days of ripening, respectively. However, Ras pH values was varied according to strains used and ranged between 5.5 ± 0.4 at day 1 to 5.2 ± 0.2 after 90 days. Overall, the achieved Ras cheese physicochemical conditions were close to literature studies and the reported results as described in section 2.2.3.

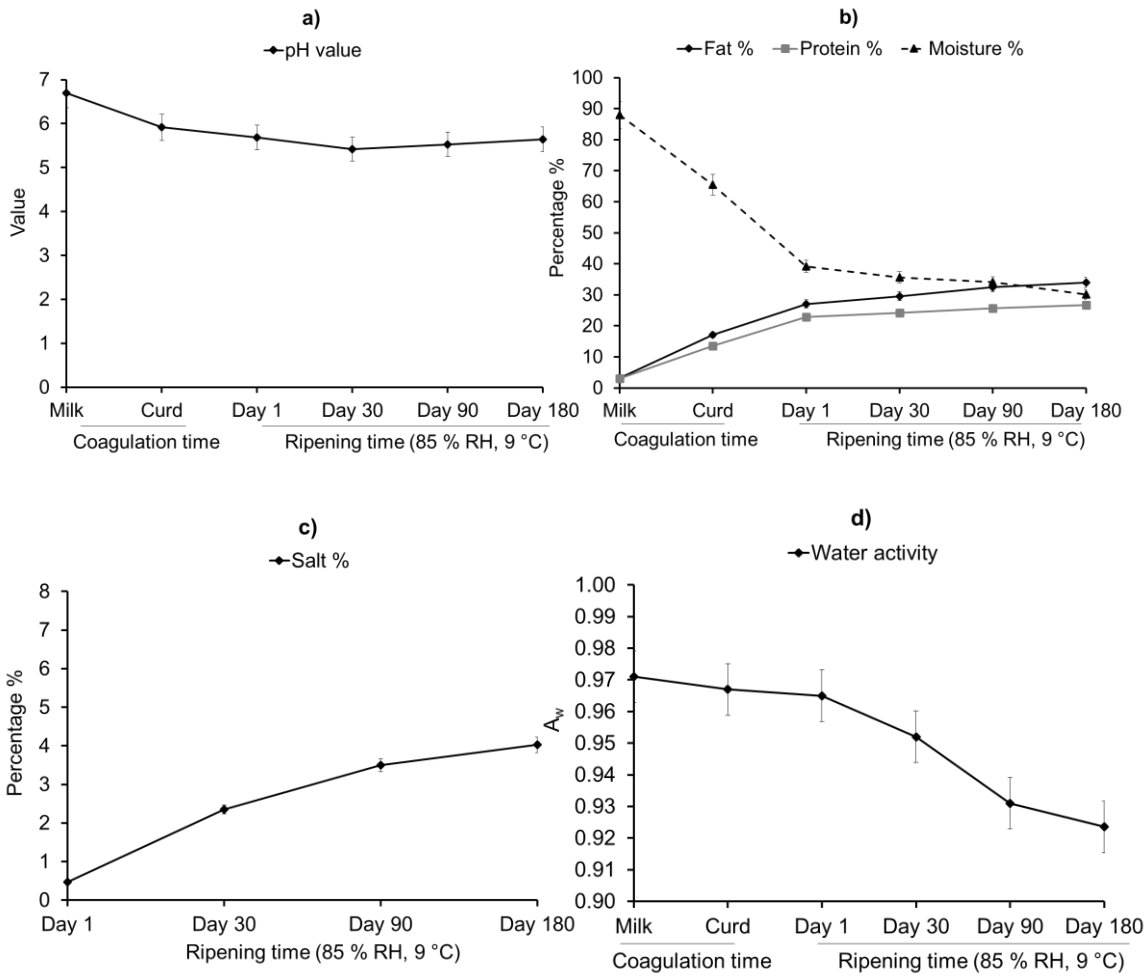

Figure 5.5. Changes in Ras cheese a) pH value; b) fat, protein and moisture %; c) salt concentration %; and d) water activity (A_w) during coagulation and ripening at 9 - 12 °C. Data reported are means ± standard deviations of two independent trials.

5.3.4 The rheological properties of Karish, Domiati and Ras cheeses

Cheese texture defined as a composite of sensory attributes resulting due to an integration of different physical properties perceived by the sense of sight and touch (Guinee, 2002)**.** The cheese texture plays an important role in the quality; however, cheese texture measurements are complicated. The texture of cheese depends upon the cheese composition and the biochemical changes during ripening (Fox *et al*., 2000). Therefore, there was a noticeable relationship between Karish, Domiati and Ras cheeses physicochemical conditions and texture profile analysis, as generally the hardness increased with the decrease in the moisture content and protein.

Table 5.2 showed that Karish cheese had the lowest hardness followed by Domiati; and Ras cheese had the highest hardness. Although Karish and Domiati are from soft type cheeses, there were a noticeable difference in the TPA, that could be linked to the difference between curd firmness produced from acid coagulation (Karish) and enzymatic coagulation (Domiati), as described in section 2.6.3.1.2. Notably, a positive relationship between the hardness, springiness and chewiness was found in Karish, Domiati and Ras; this observation is supported by Ayad *et al.* (2004).

In particular, achieved results were in accordance with Sameh (2016) who investigated the rheological properties of 15 traditional Karish samples collected from local market in different regions in Egypt with using similar TPA technique. Sameh (2016) found that hardness and chewiness were ranged between 128 – 174 and 739 – 1005, respectively; however, springiness and cohesiveness of Karish was 7.4 and 0.8, respectively.

Table 5.2. Texture profile analysis of Karish, Domiati and Ras cheeses.

Cheese	Hardness (g)	Cohesiveness	Springiness (mm)	Chewiness (g/s)
Karish	155 ± 42.01	0.62 ± 0.13	6.41 ± 1.22	843.64 ± 30.24
Domiati	235 ± 36.76	0.51 ± 0.17	6.82 ± 1.33	1013 ± 23.91
Ras	660 ± 50.45	0.48 ± 0.15	7.69 ± 1.65	2824 ± 44.37

Means are the results of duplicate samples with 10 readings per replicate at the end of storage period.

Awad (2011) listed the main reasons that affect curd moisture content, which subsequently influence cheese hardness and chewiness, these factors include: cheese chemical composition, pH value, bonding between casein and whey proteins, calcium content, salt concentration, fat content and manufacturing protocol. That could explain the variation between TPA results in three manufactured cheeses. In the same context, Awad (2006) studied the texture profile analysis of Ras cheese made from raw milk using similar sample size, and testing protocol. Awad reported that Ras hardness, cohesiveness, springiness and chewiness were 642, 0.5, 7.2 and 2433, respectively after 180 days of ripening 13 ± 2 °C and 80 ± 5 % RH.

These data demonstrate that manufacture technique followed was able to produce cheese close in texture to raw milk Egyptian manufactured cheeses. To the best of our knowledge that is the first study who tested the TBA of Domiati cheese.

5.3.5 The critical physicochemical conditions of raw milk Egyptian cheeses

Karish, Domiati and Ras cheese manufacture at small scale laboratory conditions, facilitated the assessment of the physicochemical conditions that phages would be exposed to in cheese production. Three critical conditions were identified: Firstly, the minimum and maximum temperature during manufacture were 4 and 45 °C respectively, passing through mainly 24 and 37 °C for room and curdling temperature. Secondly, the pH values ranged between 6.7 (milk pH) and 4.2 (Karish pH), and the pH values of the Ras and Domiati ranged between 5.2 and 5.9. Thirdly, the minimum salt concentration for Karish was 0.5 %, while salt % during Domiati manufacture was 8 % and the maximum salt content of Ras was 4 %; a 12 % salt was also selected as the maximum level in Domiati (as described in section 2.2.2.2).

The selected physicochemical conditions are not only relevant to Egyptian raw milk cheeses, but these selected conditions could also be applicable to any other cheeses with similar conditions. As Guinee and Fox (2004) stated that most cheeses typically have pH value from 4.6 to 6.5 and the salt concentration from 0.7 to 6 %.

5.4 Conclusions

Results obtained during Karish, Domiati and Ras cheeses manufacture proved the applicability and reproducibility of the lab-based technique used to produce cheese with physicochemical conditions close to the traditional one. Moreover, screening these conditions during manufacture and storage is necessary for identifying the ideal stage for phage application during cheese manufacture. In addition, survival of *E. coli* and *S. aureus* strains and phage lytic activity during manufacture and storage of each cheese type, could be predicted after further testing (section 6.3.2). It is therefore recommended that the physicochemical conditions of any cheese type or dairy product are studied from the beginning until the end of storage prior to application of bacteriophages.

Chapter Six: Determination of Minimum Inhibitory Concentration and Behaviour of Bacteriophages under Egyptian Raw Milk Cheese Physicochemical Conditions

6.1 Introduction

As discussed in Chapter 2, raw milk and associated products may act as a vehicle for foodborne pathogens such as *E. coli*, *S. aureus, L. monocytogenes*, *Campylobacter* spp. and *Salmonella* spp. (Lourenço *et al.*, 2017; Artursson *et al.*, 2018; Dell'Orco *et al.*, 2019), which are regarded among the main pathogens that cause a threat to human health (WHO, 2014; Moura *et al.*, 2018). In particular, recent concerns have arisen about emergence of antibiotic resistance pathogens in dairy environment (Godziszewska *et al*., 2018; Nikoloudaki *et al*., 2021). The antimicrobial resistance of *E. coli* and *S. aureus* was recently highlighted by the World Health Organization (WHO), raising the interest of scientists to find a novel way of controlling foodborne pathogens; besides, organic farms are not allowed to use antibiotics.

Bacteriophages has been shown to be a promising alternative tool for pathogen control (Chang *et al.*, 2019). Several studies have tested phages as biocontrol agents in dairy products including raw milk (Porter *et al.*, 2016), fresh cheese (Bueno *et al.*, 2012), red smear cheese (Guenther and Loessner, 2011) and cheddar cheese (El-Haddad *et al.*, 2016). However, most phage application studies in dairy environments have only been carried out in a limited range of physicochemical conditions (minimum pH 5.4 and maximum salt content 4 %).

While most cheeses typically have pH values and salt concentrations ranging from 4.6 to 6.5 and 0.7 to 6 %, respectively (Guinee, 2004). This suggests that phage efficacy needs to be evaluated under more challenging and wide-ranging physicochemical conditions.

On the other hand, several researchers identified that increasing phage concentration would increase phage treatment effectiveness against target pathogen (Guenther and Loessner, 2011; Bueno *et al.*, 2012). Vipra *et al.* (2013) argued that the lowest concentration of phages at which the highest inhibition of the target strain occurs, should be identified in each phage treatment, which would reduce cost, labour and time required.

Studies on the prevalence of *E. coli* and *S. aureus* strains in Egyptian raw milk cheeses as described in literature (section 2.3) found that the typical contamination levels of both pathogenic *E. coli* and *S. aureus* in raw milk are between 10^3 and 10^6 CFU/ml, whereby they may cause illness at levels within or greater than 10^2 and 10^5 CFU/g (FDA, 2020). In addition, these pathogens showed a high ability to remain viable during cheese manufacture and storage at those infectious concentrations (Frozi *et al.*, 2015). The complexity of cheese manufacture and varied physicochemical conditions emphasise the need to study the influence of different physicochemical conditions on phage success as biocontrol agent and to test the minimum phage concentration required to inhibit the target pathogen (Hu, Meng and Liu, 2016; García-Anaya *et al.*, 2019).

Therefore, the objectives of this work (phase 3, section 3.1) are:

i) examine the relationship between four *E. coli* and four *S. aureus* phages to control the growth of host strains at varying concentrations;

ii) evaluate the efficacy of phages to combat host strains under different temperature, pH and salt concentrations relevant to Karish, Domiati and Ras cheeses manufacture (Chapter 5).

6.2 Experimental Setup

6.2.1 Study design

This study was designed to characterise phages by determining the minimum phage concentration required to inhibit different host concentration levels and their host ranges. After that, the ideal phage concentration was selected and tested, to understand the behaviour of phages and their respective host strain under the critical physicochemical conditions of raw milk Egyptian cheeses (phase 3; section 5.3.5).

Firstly, four *E. coli* and four *S. aureus* phages were tested at four initial concentrations between 10^3 and 10^6 PFU/well against four initial host strain concentrations between 10^3 and 10^6 CFU/well. Secondly, the efficacy of phages in combatting host strains was tested under different levels of temperature (4, 24, 37 and 45 °C), pH (4.2, 5.1, 5.9 and 6.7) and salt concentration (0.5, 4, 8, 12 %). Thirdly, phage host ranges were also tested, in order to select the ideal *E. coli* and *S. aureus* phages to be tested in a dairy environment (Chapters 7 and 8).

6.2.2 Materials & methods

6.2.2.1 Bacteriophages, host strains and culture conditions

Four *E. coli* and four *S. aureus* phages and their respective host strains (Table 3.3) were cultured as described in section 3.4. The high-titre phage suspensions were prepared as described by Sambrook and Russell (2011) in section 3.4.1. The phage titre was determined by the overlay plaque assay as described by Sambrook and Russell (2011) in section 3.4.2.

The *E. coli* and *S. aureus* host stains were cultured as described in sections 3.3.1 and 3.3.2, respectively. To prepare broth cultures of host strains for microtitre plate assay, each bacterial strain was streaked on to a Nutrient agar (NA) plate (LAB M., UK) and incubated at 37 °C for 18 h. A single colony from each NA streak plate was then inoculated into Luria Bertani (LB) Broth (Fisher; US) and incubated at 37 °C for 24 h.

6.2.2.2 Determination of phage lytic ability

A microtitre plate assay was used to determine the phage lytic ability against respective host strains in sections 6.2.2.3 and 6.2.2.4 as described by Xie, Wahab and Gill (2018). Each 24 h LB broth culture was 10-fold serially diluted in SM buffer to obtain the desired concentration in each well. A standard plate count was performed using NA (Fisher; US) to check the resulting CFU/ml of the dilutions. A volume of 200 µl of LB broth was inoculated with 25 µl of 10-fold serially diluted phage and 25 µl of 10-fold serially diluted host culture strain in untreated 96-well sterile transparent plastic plates (Thermo-scientific, UK) in triplicate. The plates were incubated at the relevant temperature (according to the experiment) with orbital shaking in a BMG LABTECH plate reader (Omega 415-1305, Ortenberg, Germany) and growth was monitored by measuring OD_{600nm} with 48 readings during the testing time. Media-only, media-plus-phage and media-plus-host controls representing the normal growth of the host strain without phage inoculation were also included to calculate phage lytic ability % (section 6.2.2.5). All assays were performed in duplicate with triplicate readings.

6.2.2.3 Minimum inhibitory concentration of phage to host strain

The phage lytic ability at different phage concentrations (10^3, 10^4, 10^5 and 10^6 PFU/well) against host strain (10^3, 10^4, 10^5 and 10^6 CFU/well) concentrations were examined by microtitre plate assay (section 6.2.2.2) to determine the lowest phage concentration required to give the highest lytic ability, resulting in the highest host strain inhibition. Starting concentrations of 10^5, 10^6, 10^7 and 10^8 PFU or CFU/ml were applied to achieve final concentrations of 10^3 to 10^6 for each phage and host strain per well.

6.2.2.4 Influence of physicochemical conditions on phage lytic ability and host strain growth

The effects of previously selected critical physicochemical conditions of raw milk Egyptian cheeses (section 5.3.5) on *E. coli* and *S. aureus* host strains and the efficiency of phage treatment were examined by microtitre plate assay (section 6.2.2.2).

Firstly, the effect of temperature on *E. coli* and *S. aureus* phage and host strains was tested, using LB broth with pH 7.5 and 0.05 % salt concentration and incubated separately at 4, 24, 37 and 45 °C for 7 days, 48 h, 24 h and 12 h, respectively. Secondly, the effect of pH on *E. coli* and *S. aureus* phage and host strains was tested, using LB broth with 0.05 % salt concentration and the pH was artificially adjusted prior to sterilisation using HCl (1M, Fisher; US) to 4.2, 5.1, 5.9 and 6.7. Thirdly, the effect of salt concentration on *E. coli* and *S. aureus* phage and host strains was tested, using LB broth with pH 7.5 and salt concentration was artificially adjusted prior to sterilisation using NaCl to 0.5, 4, 8 and 12 %.

Initial bacterial and phage inoculum levels of 10^7 CFU/ml and 10^7 PFU/ml, respectively, were employed to achieve a final concentration of 10^5/well for each phage and host strain (based on the upper limit for coagulase positive staphylococci in cheese) (Commission Regulation [EC] No., 2073/2005) and the typical contamination levels reported in raw milk Egyptian cheeses.

6.2.2.5 Analysis of microtitre assay data

To monitor growth during the assay, the phage lytic ability was calculated. The results achieved from microtitre assay in sections 6.2.2.3 and 6.2.2.4 were analysed, firstly using equation 1 to calculate the area under each curve and then the phage lytic ability was calculated using equation 2 (Xie, Wahab and Gill, 2018). An illustration of the lytic ability calculation, which represents how well phages are able to suppress bacterial growth during the 24 h experiment, is shown in Figure 6.1.

$$\text{Area under curve} = \sum_{i=1}^{49} (OD_{i+1} + OD_i)/2 \qquad \text{(Equation 1)}$$

where OD was measured at 600 nm at interval time points i

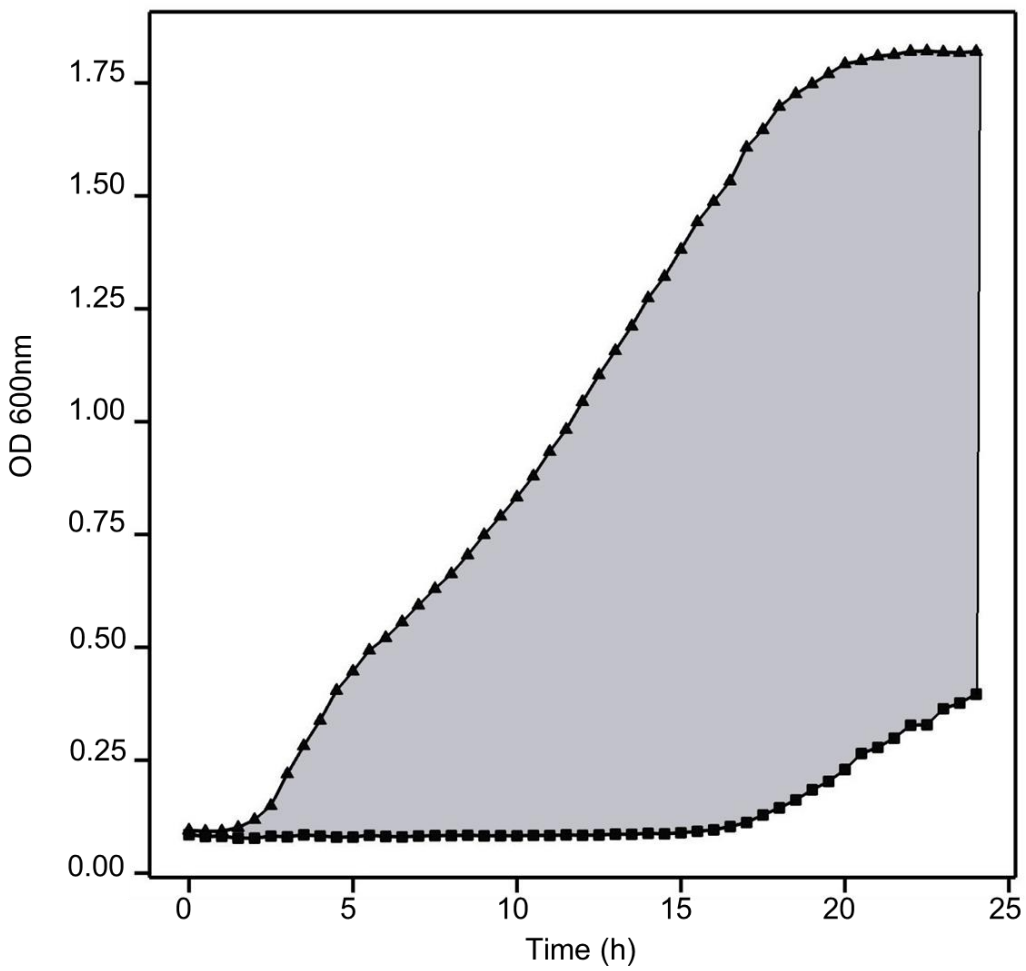

Figure 6.1. An illustration of phage lytic ability (grey area) calculated by Equation (2) between the negative control (▲) and the positive control (■) and expressed as a percentage of the total area between negative control (host-strain-only) and treatment (phage plus host strain) area.

$$\text{Phage lytic ability \%} = \frac{\text{Area}_{\text{Negative control}} - \text{Area}_{\text{Positive control}}}{\text{Area}_{\text{Negative control}}} * 100 \qquad \text{(Equation 2)}$$

6.2.2.6 Determination of phage host range

The host ranges of *E. coli* and *S. aureus* phages (Table 3.3) were determined by spot test assay in duplicate using a variety of different bacteria (Table 3.2) and previously isolated *E. coli* and *S. aureus* strains from raw milk. Using the overlay technique (section 3.4.2) a lawn of each strain was prepared and the plate was then divided into four sections. After that, 3 µl of 10-fold diluted phage stock at 10^8 PFU/ml were spotted onto the prepared plate (Figure 6.2a and b) and incubated at 37 °C for 18 – 24 h.

MacConkey agar medium (Figure 6.2b) was used to grow only the non-pathogenic *E. coli* surrogates (Table 3.2). The host range of the phages was assessed on each tested strain by a common scoring system (Huang *et al.*, 2018) (Figure 6.2).

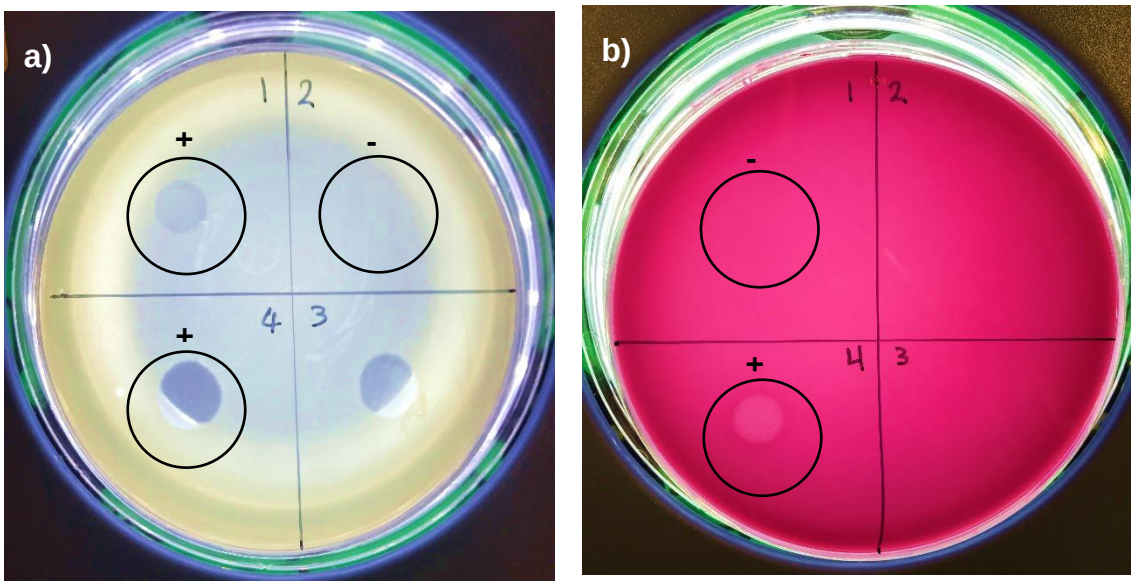

Figure 6.2. An illustration of the host range determination in LB agar medium (a) and MacConkey agar medium (b) using a common scoring system as follows: (+), complete lysis; (±), growth inhibition; (-), no growth inhibition.

6.2.3 Statistical analysis

Results are presented as phage lytic ability percentage means ± standard deviation (n = 6). A general design analysis of variance (ANOVA) was carried out to determine any significant differences ($p \leq 0.05$) among the treatments and Duncan's multiple range test ($p \leq 0.05$) was applied to compare the average values obtained between control and treatment using GenStat software (GenStat V 18.1. 17005, England, UK).

6.3 Results & Discussion

6.3.1 Determination of phage minimum inhibitory concentration

6.3.1.1 MIC of phages to control *E. coli*

The lytic ability results for four *E. coli* phages at 10^3 to 10^6 PFU/well against 10^3 to 10^6 CFU/well host cell concentration are shown in Figure 6.3. Across all phage and cell concentrations interactions, *E. coli* T4 showed a higher significant ($p \leq 0.05$) ability to inhibit host strain growth, by 96.21 %, than *E. coli* K1E (48.21 %), EcoM (42.04 %) and EcoS (15.88 %). On the other hand, for all phage concentrations, growth of a 10^3 CFU/well cell concentration was suppressed by 63.76 % (p= 0.006), followed by 47.29 % at 10^4 CFU/well and 46.30 % (p= 0.552) at 10^5 CFU/well, and by 45.00 % ($p \leq 0.05$) when the cell concentration increased to 10^6 CFU/well. This demonstrates the higher phage efficacy to reduce or eliminate the host strain at lower cell concentrations.

These findings are in concurrence with Kasman *et al.* (2002), who concluded from a mathematical model that phage replication happens at any host cell density between 10^1 and 10^9 CFU/ml, proving that even at low host cell densities, bonding between phage and bacterial cells can occur if there are sufficient numbers of phages. However, Wiggins and Alexander (1985) stated that the minimum bacterial threshold density for phage replication is 10^4 CFU/ml using Trypticase soy broth at 28 °C. Wiggins and Alexander focused on testing *E. coli* T4, *S. aureus* 80α and *B. subtilis* SPβ phages against host strains (*E. coli* SH305, *S. aureus* RN450 and *B. subtilis* CU3389) at three different concentration levels (10^3, 10^5 and 10^6 CFU/ml) at 28 °C, where they found that phages required between 90 and 160 mins to begin to increase rapidly at all cell concentrations. This implies that phage replication is affected in particular by the host growth state, not the density of the host.

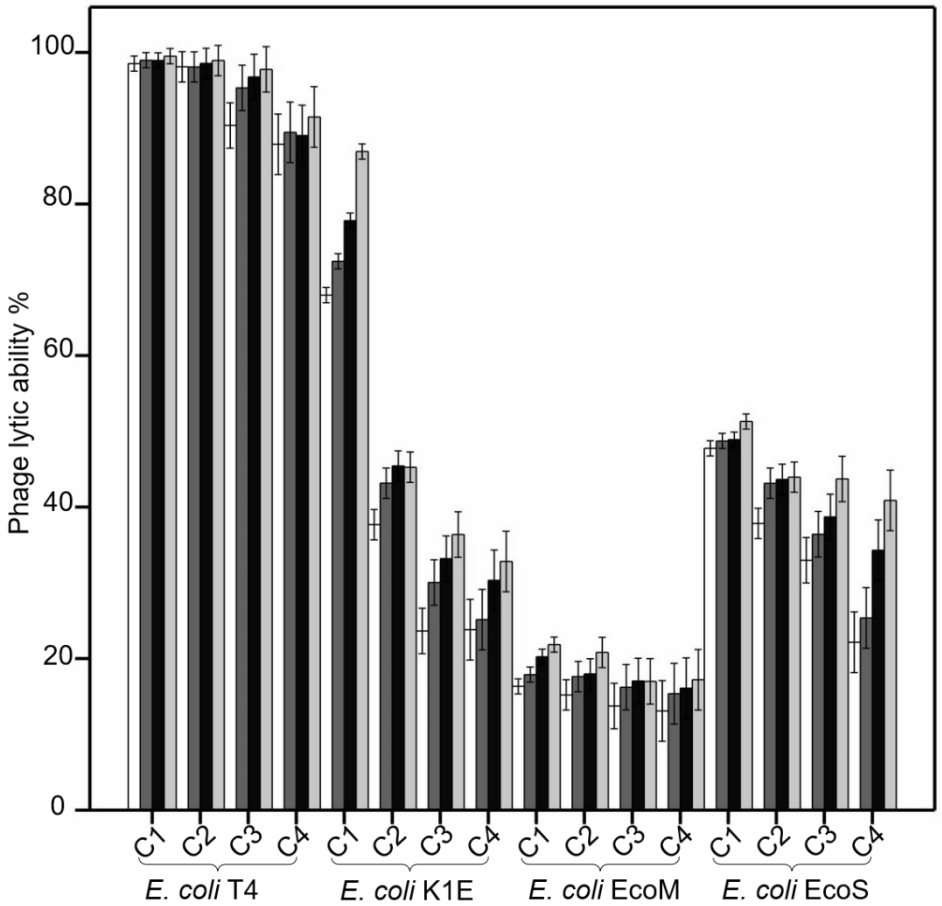

Figure 6.3. Phage lytic ability of *E. coli* T4, K1E, EcoM and EcoS incubated at 37 °C for 24 h using microtitre plate assay, using cell concentration 10^3 to 10^6 CFU/well with 10^3 (▭), 10^4 (■), 10^5 (■) and 10^6 (■) PFU/well phage concentration. Lytic ability percentages are mean values based on the results from the four tested cell concentrations (C1 = 10^3, C2 = 10^4, C3 = 10^5 and C4 = 10^6 CFU/well). Data reported are means ± standard deviations (error bars) following six replications.

Moreover, these findings reflect the high potential of *E. coli* T4 to inhibit the host strain within all tested phage concentrations. This is in concurrence with Liu *et al.* (2015) who found that *E. coli* T4 was the most efficacious phage to reduce *E. coli* cell numbers by 1.5 log CFU/ml at 37 °C after 3 h of incubation. This may explain why T4 is one of the most common phages used to control pathogenic *E. coli* (Carter *et al.*, 2012; Śliwka *et al.*, 2019).

The observed differences in phage lytic ability with the interaction between phage concentrations for *E. coli* T4, K1E, EcoS and EcoM, can be indicated to determine the minimum phage required to control each host strain. These data revealed that using 10^6 PFU/well showed the highest phage lytic ability, at 51.27 %, within four cell concentrations, and there was no significant difference (p= 0.721) between using 10^6 or 10^5 PFU/well phage concentration. In contrast, using 10^3 PFU/well showed the lowest phage lytic ability, at 50.02 %.

6.3.1.2 MIC of phages to control *S. aureus*

The four *S. aureus* phage lytic ability results at 10^3 to 10^6 PFU/well against 10^3 to 10^6 CFU/well host cell concentration, are illustrated in Figure 6.4. *S. aureus* K2 and EBHT showed the highest ability to reduce host strain growth, by 97.72 and 89.29 %, respectively, and were significantly different ($p \leq 0.05$) from *S. aureus* CS1 (54.01 %) and K1 (53.23 %).

On the other hand, within all *S. aureus* phage concentrations, the phage lytic ability at 10^3 CFU/well was higher (p= 0.003), at 81.57 %, and decreased to 66.11 % when the cell concentration increased to 10^6 CFU/well, which was similarly noticed with *E. coli* phages. In accordance with the *E. coli* phage results, 10^6 PFU/well phage concentration showed the highest ($p \leq 0.05$) ability to inhibit host strain, by 84.45 % within four cell concentrations, and this decreased to 60.67 % at 10^3 PFU/well. This shows that the higher the phage concentration, the more effective the control of the host strain, which can be attributed to the increase of interaction probability between bacterial cells and phage, that implement the effectiveness of target pathogen biocontrol (Hagens and Loessner, 2010; Gouvêa *et al.*, 2016).

It is critical to understand that phage thresholds and behaviours in different food matrices are completely different. Hagens and Loessner (2010) argue that it is the phage concentration that must be high enough to enable sufficient contact and subsequent infection of the target strain, even when the target strain is at low level of concentration. They added that it is important for the phage threshold concentration to be sufficiently high to spread into the free space within the food matrix.

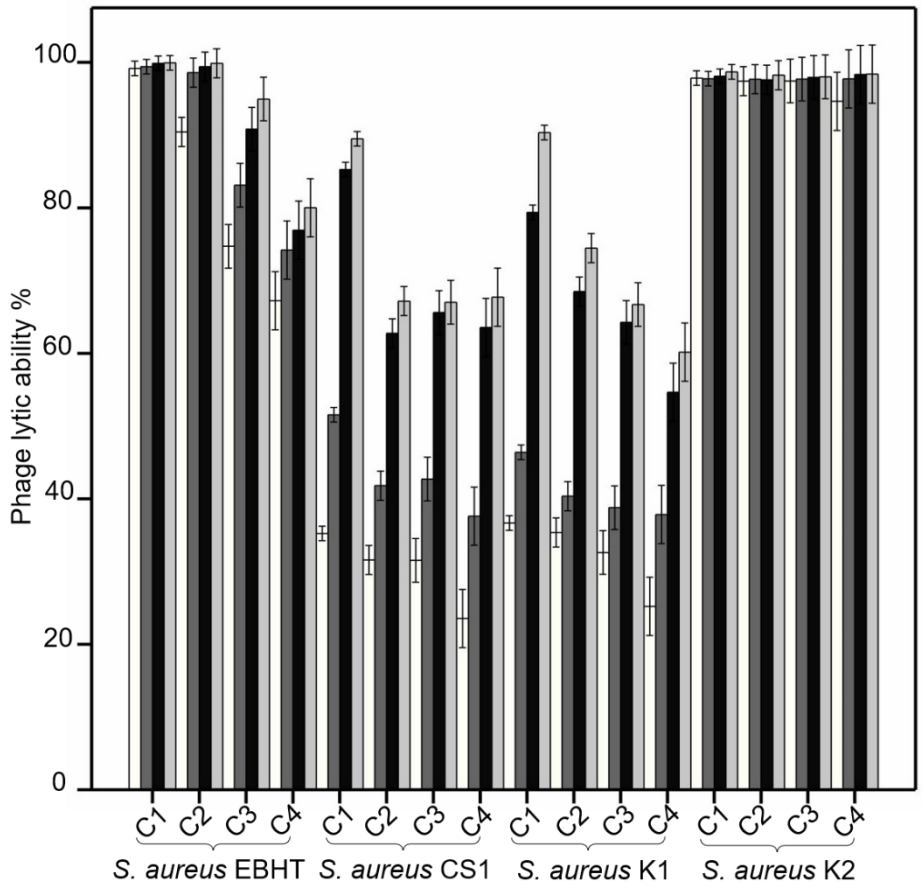

Figure 6.4. Phage lytic ability of *S. aureus* EBHT, CS1, K1 and K2 incubated at 37 °C for 24 h using microtitre plate assay, using cell concentration 10^3 to 10^6 CFU/well with 10^3 (▭), 10^4 (■), 10^5 (■) and 10^6 (■) PFU/well phage concentration. Lytic ability percentages are mean values based on the results from the four tested cell concentrations (C1 = 10^3, C2 = 10^4, C3 = 10^5 and C4 = 10^6 CFU/well). Data reported are means ± standard deviations (error bars) following six replications.

Furthermore, several studies have used high phage concentrations between 10^8 and 10^{10} PFU/ml to give better control of the host strain at 10^6 CFU/ml (García *et al.*, 2007; Yeh *et al.*, 2017). However, some researchers have reported reduced phage inhibition when the cell concentration is below 10^6 CFU/ml, and a higher phage to host strain ratio is therefore required (Kasman *et al.*, 2002; Guenther *et al.*, 2012). Ultimately, determination of the minimum concentrations required to control the host strain will result in a more cost and time effective phage application (Vipra *et al.*, 2013). These observations highlight the importance of MIC determination for selected phages to ensure the highest biocontrol activity with the most efficient application.

As the results varied between phages, and the lytic ability difference between 10^6 and 10^5 PFU/well phage concentration was not significant (p= 0.752) for *S. aureus* phages EBHT and K2, the MIC of 10^5 PFU/well was selected to determine phage lytic ability under Egyptian raw milk cheeses physicochemical conditions against host strains.

6.3.2 Influence of Egyptian raw milk cheese physicochemical conditions on *E. coli* and *S. aureus* strains growth and phage lytic ability

The phage lytic ability of four *E. coli* and four *S. aureus* phages at different physicochemical conditions (temperature, pH and salt) are illustrated in Table 6.1. The achieved results revealed that *E. coli* T4, *S. aureus* EBHT and *S. aureus* K2 showed the highest lytic ability between 100 and 95.5 % at 24 and 37 °C. However, at 45 °C, the ability of all *E. coli* and *S. aureus* phages to inhibit host strain growth was decreased significantly ($p \leq 0.05$), with part of this effect related to the observed lower ability of *S. aureus* host strains to grow at 45 °C. Furthermore, there was no recorded growth of *E. coli* and *S. aureus* host strain at 4 °C. These findings revealed that targeting and eliminating host strains was more efficient at median temperature. This is in accordance with Liu *et al.* (2015) who tested four phages (T5, T1, T4 and O1) on beef at 10^6 and 10^8 PFU/cm^2 against *E. coli* O157 at 10^5 CFU/cm^2 and found that phages were more effective at 37 °C than at 4 and 22 °C. Additionally, Chang *et al.* (2019) found that SA13m phage (10^7 PFU/ml) was able to reduce *S. aureus* count (10^5 CFU/ml) to non-detectable levels in milk at 25 °C after 24 h, while at 4 °C a 4.1 log CFU/ml reduction was achieved.

The differences in lytic ability results for *E. coli* and *S. aureus* phages under the four tested temperatures could be attributed to the optimum growth temperature (around 35 to 42 °C) for the *E. coli* and *S. aureus* strains (Kudva *et al.*, 1999), as well as phage stability and adsorption rate to host cells. Tomat *et al.* (2014a) reported that phage adsorption in Hershey-Mg broth was decreased at 4 and 50 °C, while phage adsorption reached the highest rate at 37 °C. Moreover, the phage receptors on the bacterial cell surface could be partially denaturated at high temperature, while the receptors may be segregated into patches at low temperatures (below 10 °C) (Moldovan, Chapman-McQuiston and Wu, 2007).

Table 6.1. Phage lytic ability percentages of four *E. coli* and four *S. aureus* phages using microtitre plate assay at four different temperatures, pH values and salt concentrations.

Treatment / Strain		*E. coli* phage				*S. aureus* phage			
		T4	K1E	EcoS	EcoM	EBHT	CS1	K1	K2
Temperature °C	45	69.38^{e*}	19.37^{b}	9.37^{a}	8.29^{a}	12.89^{c}	3.39^{a}	7.54^{b}	N.D.
	37	99.92^{f}	33.51^{c}	44.81^{d}	42.95^{d}	99.50^{g}	63.35^{e}	63.54^{e}	100.00^{g}
	24	99.52^{f}	23.29^{b}	34.59^{c}	42.36^{d}	98.45^{g}	51.34^{d}	61.34^{e}	95.50^{f}
	4	N.D.**	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
pH	6.7	99.62^{i}	35.11^{ef}	46.41^{h}	44.49^{gh}	99.73^{e}	65.15^{d}	67.00^{d}	100.00^{e}
	5.9	99.48^{i}	32.36 ^{de}	43.66^{gh}	36.04^{f}	99.26^{e}	65.61^{d}	65.80^{d}	100.00^{e}
	5.1	99.71^{i}	31.58^{d}	42.88^{g}	35.49^{f}	94.74^{e}	55.35^{c}	63.54^{d}	36.88^{b}
	4.2	32.12^{de}	12.03^{b}	23.33^{c}	0^{a}	0^{a}	0^{a}	0^{a}	N.D.
Salt %	12	N.D.	N.D.	N.D.	N.D.	89.54^{f}	48.19^{c}	46.71^{c}	0^{a}
	8	N.D.	N.D.	N.D.	N.D.	99.41^{g}	46.25^{c}	66.30^{e}	20.43^{b}
	4	68.84^{d}	N.D.	N.D.	45.76^{c}	99.52^{g}	63.83^{de}	67.35^{e}	60.50^{d}
	0.5	99.65^{f}	35.92^{a}	47.22^{c}	42.69^{b}	99.86^{g}	63.84^{de}	65.70^{e}	99.86^{g}

*Lytic ability percentages are mean values based on the results from the four tested cell concentrations. Data reported are means ± standard deviations. For clarity the phage lytic ability percentages were classified to 100 – 80 % (■), 79 – 60 % (■), 59 – 40 % (■) and ≤ 40 % (□). **N.D.: Not detected, the lytic ability wasn't able to be detected in samples where the host strain showed no growth under tested conditions. Effect of temperature was done at pH 7.5 and 0.05 % salt. Effect of pH was done at 37 °C and 0.05 % salt. Effect of salt was done at 37 °C and pH 7.5.

However, Coffey *et al.* (2011) found that two phages (e11/2 and e4/1c) tested against *E. coli* O157 were stable under a wide range of temperatures (-22 to 60 °C) in LB broth media. On the other hand, Moldovan, Chapman-McQuiston and Wu (2007) investigated the coliphages λ receptor binding at 4 and 37 °C using fluorescent microscopy, their obtained results showed a uniform phage distribution on bacterial cell wall at 37 °C, and phage aggregated in small patches at 4 °C, however, the link between recorded reduction in adsorption and lytic ability was not clear.

The phage lytic ability results for the four *E. coli* phages at different pH values (Table 6.1) showed no significant differences at pH 5.1, 5.9 and 6.7 for all phages, while the lytic activity decreased significantly ($p \leq 0.05$) at pH 4.2. However, there were no significant differences between *S. aureus* phage lytic ability at pH 5.9 and 6.7; *S. aureus* phage ability to suppress host strain growth was decreased significantly at pH 5.1 and no effect recorded at pH 4.2. This could be attributed to the lower ability of phages to adsorb to host strains at low pH, a phenomenon previously observed by Tomat *et al.* (2014b) who found that, at pH values between 5.7 and 7.5, the adsorption rate of three *E. coli* phages (DT1, DT5 and DT6) was around 96 %, whereas at pH 4.5 the adsorption rate decreased to 55 %.

In addition, lowering the pH value decreases the *E. coli* host strain growth rate (Figure 6.5) as the lag phase increases; there are no noticeable differences between the growth rate of *S. aureus* host strain (Figure 6.5) at pH 5.9 and 6.7, while lowering the pH to 4.2 had a high negative effect on the growth rate. *E. coli* host strain growth showed that it can withstand low pH more than *S. aureus* host strain. Similar observations have been stated by many authors, that *E. coli* is able to grow over a wide range of pH values from 4.5 to 9 (Elhadidy and Mohammed, 2013; Callon *et al.*, 2016) and can survive acidic conditions down to pH 4 for around 56 days (Kotrola, 1995).

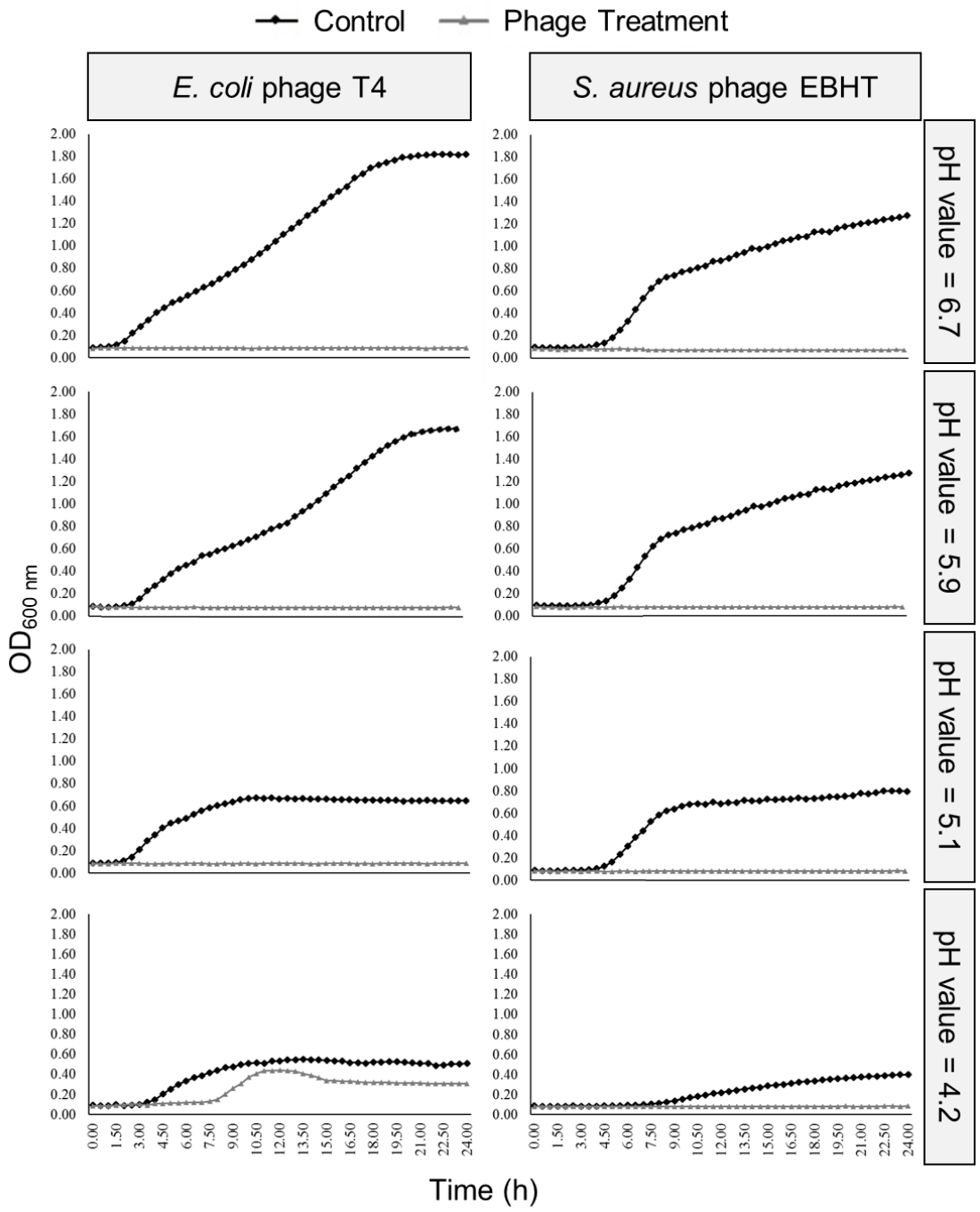

Figure 6.5. Sample growth curves of host-strain-only (control) and host-strain plus phage (phage treatment, 10^5 PFU/well) using LB medium under different pH conditions (6.7, 5.9, 5.1 and 4.2 value) and 0.05 % salt. Optical densities ($OD_{600\ nm}$) were measured at 37 °C for 24 h using microtitre plate reader (n= 3) with 30 min interval time. *E. coli* NCIMB 10243 or *S. aureus* DSM 105272 (10^5 CFU/well) was used as a host strain for each respective phage.

The lytic ability of four tested *E. coli* phages at different salt concentrations (Table 6.1), revealed that phages were able to show higher significant ($p \leq 0.05$) inhibition of host strain at 0.5 % salt than at 4 % salt. Furthermore, *E. coli* host strains were not able to grow at 8 and 12 % salt (Figure 6.6), as *E. coli* survives only at salt concentrations ranging from 1 to 6 % (Elhadidy and Mohammed, 2013). In contrast, there was no significant difference ($p \leq 0.05$) in phage lytic ability for *S. aureus* EBHT, CS1 and K1 at 0.5 and 4 % salt, while lytic ability at 8 and 12 % salt was significantly reduced. Furthermore, *S. aureus* host strains showed the ability to grow at 8 and 12 % salt concentration (Figure 6.6). However, *S. aureus* K2 showed the highest lytic ability at pH 5.9, 6.7 and 0.5 % salt, but was highly reduced at pH 5.1 or 8 and 12 % salt.

The possible explanations of the difference in phage effect on host strains could firstly be attributed to the increase in salt concentration increasing the osmotic pressure, which has a negative effect on the host strain growth (Omotoyinbo and Omotoyinbo, 2016); secondly, Sodium ions (Na+) have a negative effect on the phage adsorption rate by replacing cations such as Ca^{2+} which destabilise the adsorption between phage and the host strain (Bandara *et al.*, 2012; Tomat *et al.*, 2014a). Although *S. aureus* K2 showed lower lytic ability at more challenging physicochemical conditions; many researchers have observed phage K broad host range against strains of human and bovine origin (O'Flaherty *et al.*, 2005a; Gutiérrez *et al.*, 2016), that could make phage K a good candidate to control *S. aureus* in dairy application.

Overall, achieved data demonstrate that *E. coli* T4, *S. aureus* EBHT and *S. aureus* K2 showed the highest host strain inhibition rate within the three tested physicochemical conditions. In addition, *E. coli* host strains used would be able to survive during manufacture until the end of storage of cheeses with acidic conditions such as Karish and Ras cheese. However, cheeses with high salt concentration, such as Domiati and to some extent Ras, are more susceptible to *S. aureus* survival during manufacture and storage, which is attributed to the recorded growth at 8 and 12 % salt. An understanding of phage and host strain behaviour at tested physicochemical conditions is therefore essential to set the ideal protocol with high phage efficacy to control *E. coli* and *S. aureus* in raw milk cheeses.

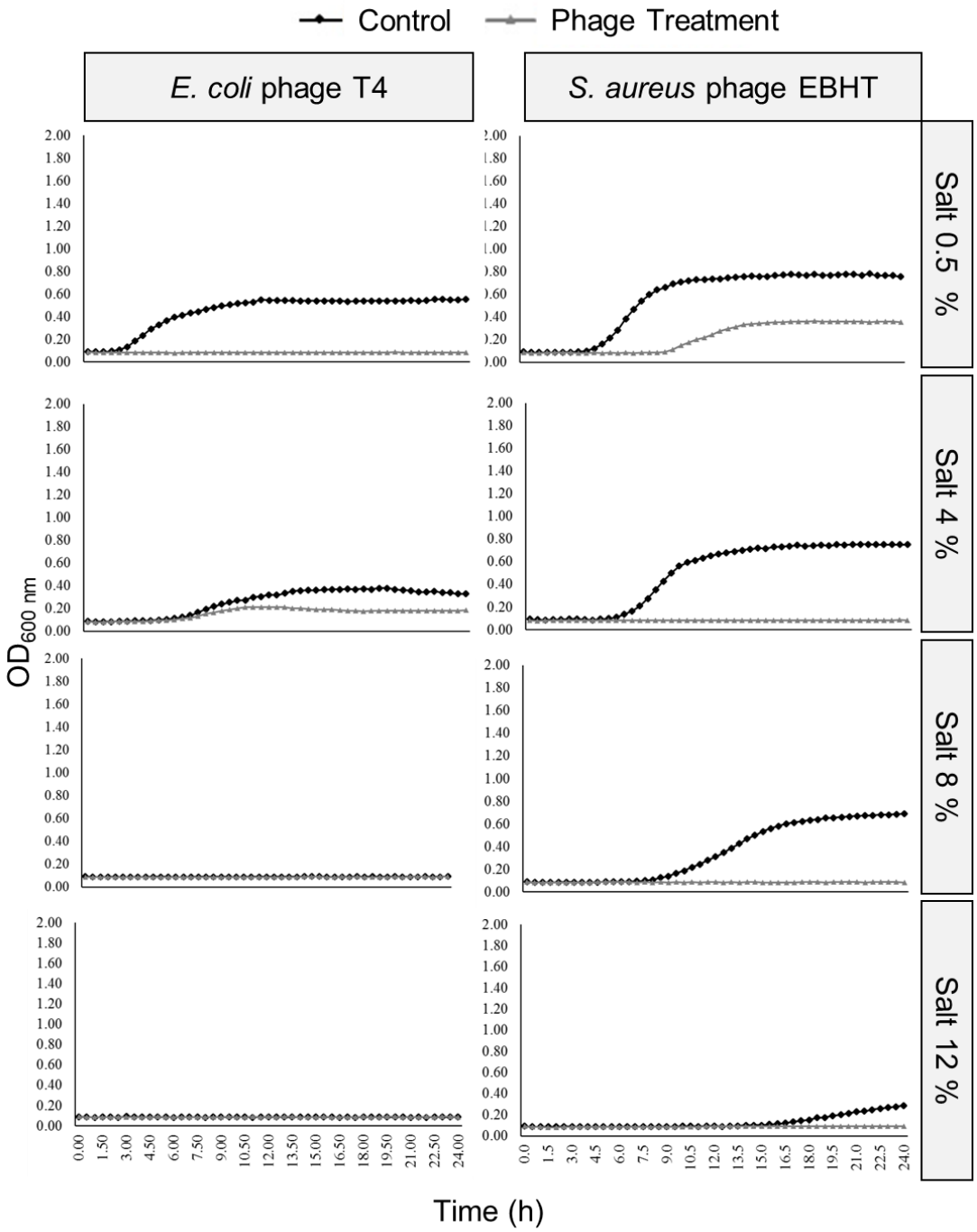

Figure 6.6. Sample growth curves of host-strain-only (control) and host-strain plus phage (phage treatment, 10^5 PFU/well) under different salt concentrations (0.5, 4, 8 and 12 %) and pH 7.5. Optical densities ($OD_{600\ nm}$) were measured at 37 °C for 24 h using microtitre plate reader (n= 3) with 30 min interval time. *E. coli* NCIMB 10243 or *S. aureus* DSM 105272 (10^5 CFU/well) was used as a host strain for each respective phage.

6.3.3 Determination of phages host range

The host range results of four *E. coli* phages against reference and previously isolated (section 4.3.1) *E. coli* strains, are shown in Table 6.2. Phage T4 and EcoM showed wider host range compared to other tested phages, due to their ability to target various host specificity. Phage T4 was able to target two *E. coli* reference strains and four *E. coli* isolates, while phage EcoM was able to target one non-pathogenic *E. coli* surrogate and three *E. coli* reference strains. Furthermore, the four *E. coli* phages did not target the other Gram-negative strains, Gram-positive strains and LAB strains (Table 6.3).

Host range characterization of the four *S. aureus* phages was performed with seven reference and twenty isolated *S. aureus* (section 4.3.2) strains (Table 6.4) and also three Staphylococcal strains (representing 3 serovars) (Table 6.5). Phage EBHT, K1 and K2 showed a wide host specificity against most of reference and isolated *S. aureus* strains. Interestingly, phage K1 and K2 were able to targ*et a*ll other Staphylococcal spp., while EBHT was able to target one staphylococcal spp. in addition, the four *S. aureus* phages did not target the other Gram-negative strains, non-Staphylococcal Gram-positive strains and LAB strains (Table 6.5).

Host range determination is one of the main criteria to select a lytic phage to control target strains (Guyader and Burch, 2008). Phages showing a broad lytic range are potentially used as biocontrol agent of bacterial contamination in foods (Hoang *et al.*, 2016). Phage wide host range within mainly same species give the advantage of: a) reduce time spent to face the host, b) could replicate in non-pathogenic bacteria (Bielke *et al.*, 2007), c) facilitate the process of phage treatment by using single phage instead of mixture of various phages (Carlton *et al.*, 2005), d) these advantages could subsequently reduce cost in phage production (Hungaro *et al.*, 2013). That indicate the necessity of host range determination in order to select the ideal phage to be applied in raw milk cheese manufacture.

The inability of all *E. coli* and *S. aureus* phages to target LAB strains, previously isolated from Egyptian dairy products (Awad *et al*., 2007), demonstrates their ability to eliminate only their target bacteria without affecting raw milk natural microbiota.

Table 6.2. Evaluation of *E. coli* phage host ranges against *E. coli* strains by using spot test assay.

Strain		*E. coli* phage			
		T4	**K1E**	**EcoS**	**EcoM**
***E. coli* reference strains**					
Non-pathogenic surrogate	BAA-1427	-	-	-	-
	BAA-1428	-	-	-	-
	BAA-1429	-	-	-	±
	BAA-1430	-	-	-	-
	BAA-1431	-	-	-	-
DSM 22665		+	-	-	±
NCIMB 10243		+	-	-	±
DSM 101113		-	+	+	-
DSM 106579		-	-	-	+
***E. coli* isolated strains**					
3760		-	-	-	-
2254		-	-	-	-
3748		+	-	-	-
3394		-	-	-	-
3198		-	-	-	-
3709		-	-	-	-
102		+	-	-	-
4024		-	-	-	-
3977		-	-	-	-
4291		-	-	-	-
3301		-	-	-	-
4022		+	-	-	-
212		+	-	-	-
3761		-	-	-	-
2259		-	-	-	-
2958		-	-	-	-

(+), complete lysis; (±), growth inhibition; (-), no growth inhibition

Table 6.3. Evaluation of *E. coli* phage host ranges against different non-*E. coli* species by using spot test assay.

Strain	*E. coli* phage			
	T4	**K1E**	**EcoS**	**EcoM**
Other Gram-negative species				
K. pneumoniae DSM 789	-	-	-	-
Y. enterocolitica DSM 11067	-	-	-	-
E. hermanni DSM 4560	-	-	-	-
Gram-positive species				
S. aureus ATCC 25923	-	-	-	-
S. aureus DSM 12463	-	-	-	-
S. hyicus DSM 17421	-	-	-	-
S. epidermidis DSM 28319	-	-	-	-
S. haemolyticus DSM 20263	-	-	-	-
S. warneri DSM 20036	-	-	-	-
L. monocytogenes DSM 15675	-	-	-	-
B. coagulans DSM 1	-	-	-	-
LAB Gram-positive species				
L. helveticus FAAU 27st	-	-	-	-
Lb. paracasei subsp. paracasei FAAU 119th	-	-	-	-
En. faecium FAAU 102E	-	-	-	-
En. faecium FAAU 236E	-	-	-	-
En. faecium FAAU 241E	-	-	-	-

(+), complete lysis; (±), growth inhibition; (-), no growth inhibition

Table 6.4. Evaluation of *S. aureus* phage host ranges against *S. aureus* strains by using spot test assay.

Strain	*S. aureus* phage			
	EBHT	**CS1**	**K1**	**K2**
***S. aureus* reference strains**				
DSM 104437	+	+	+	+
DSM 105272	+	+	+	+
ATCC 19685	+	+	+	+
ATCC 33591	-	-	+	+
ATCC 25923	±	-	+	+
DSM 12463	+	+	+	+
NCTC 13552	+	-	+	+
***S. aureus* isolated strains**				
3421	+	-	+	+
2254	+	-	+	+
2958	+	-	+	+
3977	+	-	+	+
3743	+	-	+	+
3748	+	-	+	+
2933	+	-	+	+
3749	+	-	+	+
3747	+	-	+	+
4365	+	-	+	+
3741	+	-	+	+
3742	+	-	+	+
324	+	-	+	+
3983	+	-	+	+
4304	-	-	-	-
2790	+	-	+	+
3720	-	-	-	-
3516	+	-	+	+
23	+	-	+	+
3723	+	-	+	+

(+), complete lysis; (±), growth inhibition; (-), no growth inhibition

Table 6.5. Evaluation of *S. aureus* phage host ranges against different species by using spot test assay.

Strain	***S. aureus* phage**			
	EBHT	**CS1**	**K1**	**K2**
Other gam-positive species				
S. hyicus DSM 17421	±	-	±	+
S. epidermidis DSM 28319	-	-	+	+
S. haemolyticus DSM 20263	-	-	+	+
S. warneri DSM 20036	-	-	+	+
L. monocytogenes DSM 15675	-	-	-	-
B. coagulans DSM 1	-	-	-	-
LAB Gram-positive species				
L. helveticus FAAU 27st	-	-	-	-
Lb. paracasei subsp. paracasei FAAU119th	-	-	-	-
En. Faecium FAAU 102E	-	-	-	-
En. Faecium FAAU 236E	-	-	-	-
En. Faecium FAAU 241E	-	-	-	-
Gram-negative species				
E. coli DSM 22665	-	-	-	-
E. coli NCIMB 10243	-	-	-	-
K. pneumoniae DSM 789	-	-	-	-
Y. enterocolitica DSM 11067	-	-	-	-
E. hermanni DSM 4560	-	-	-	-

(+), complete lysis; (±), growth inhibition; (-), no growth inhibition

6.4 Conclusions

Phages have been shown to be effective in controlling *E. coli* and *S. aureus* strains, with determination of the minimum inhibitory concentration an essential step to reduce cost and make the application more time effective by choosing the lowest effective phage concentration. Moreover, *S. aureus* EBHT and K2 showed a wide host range against *S. aureus* and *Staphylococcus* spp.; although, *E. coli* T4 showed narrow host range, it had a greater host range and lytic ability compared to the other tested phages. Therefore, *E. coli* phage T4, *S. aureus* EBHT and K2 were selected based on their high lytic ability, host range and efficiency under varied physicochemical conditions.

These features reflect the high potential of those phages to be selected as useful candidates for controlling *E. coli* and *S. aureus* strains in Egyptian raw milk cheeses. Interestingly, the achieved phage host ranges of *E. coli* T4, *S. aureus* EBHT and *S. aureus* K2 exhibit their potential to target inoculated host and indigenous target strains. Moreover, the results of phage lytic ability under selected physicochemical conditions are critical to determine the ideal stage of phage application during cheese manufacture. However, further testing is required to test phage behaviour in the dairy environment to see the effect of milk components on phage efficacy (Chapter 7).

Chapter Seven: Evaluation of *E. coli* and *S. aureus* Bacteriophages as Biocontrol Agents in Sterilized and Raw Cow's Milk

7.1 Introduction

As discussed in Chapter 2, raw milk can be contaminated by several foodborne pathogens (Brooks *et al*., 2012; Hummerjohann *et al*., 2014; Hoque *et al*., 2018; Uyanik *et al*., 2022) while several studies have stated that *E. coli* and *S. aureus* can also survive during cheese manufacture and storage (Peng *et al*., 2013a; Mehli *et al*., 2017; Abdel-Hameid Ahmed *et al*., 2019). The most recent study on the microbiological quality of unpasteurised milk cheese in England by Willis *et al*. (2021) showed that 78 (12.4 %) out of 629 collected market samples were unsatisfactory and potentially injurious (positive for coagulase-positive staphylococci, *E. coli stx* genes and *L. monocytogenes*). These highlight the need to control foodborne pathogens mainly in raw milk, to inhibit pathogen growth and limit the chance for enterotoxins production.

Bacteriophages have been shown to be a promising tool for pathogen control (Chang *et al.*, 2019) due to a number of desired properties (Chapter 2), mainly their high specificity to target strain and generally do not cross species or genus level (Komora *et al.*, 2018). Phages are commonly found in raw milk and dairy environments, where LAB phages can create problems for cheese and fermented dairy products manufacture by suppressing starter cultures growth (Madera *et al*., 2004; Spus *et al*., 2015). However, other specific phages to foodborne pathogens could eliminate pathogens without affecting either the natural food (Bielmann *et al.*, 2015) or human commensal microbiotas (Carlton *et al.*, 2005).

In Chapter 6 tested bacteriophages were shown to be an effective tool to control *E. coli* and *S. aureus* under a wide range of challenging physicochemical conditions. However, phage success largely depends on the food structure (Guenther *et al.*, 2009). Several studies have tested phages as biocontrol agents in raw milk (O'Flaherty *et al*., 2005b; McLean *et al*., 2013; Porter *et al*., 2016), semi-skimmed raw milk (García *et al.*, 2009), pasteurized whole milk (García *et al*., 2009; Tabla *et al*., 2012; Zinno *et al*., 2014) and reconstituted skim milk (Endersen *et al.*, 2013) have stated there are many factors that could affect phage biocontrol activity in milk.

Gill *et al*. (2006) suggested that the presence of whey proteins could reduce the binding rate of *S. aureus* phage K to host strain due to interference by whey proteins. In addition, García-Anaya *et al*. (2019) provide information about the interactions between milk fat and phages. This study found a higher affinity of *Listeria* phage A511 to fat from unhomogenized milk (33.5 %) than in fat from homogenized milk (5.5 %). However, it was noted through the review of literature that little attention has been paid to the effect of milk fat separation in relation to heat treatment on the phage biocontrol activity. In addition, there is not enough evidence whether phages bonded to milk components could still be infectious or able to replicate (García-Anaya *et al*., 2019; García-Anaya *et al*., 2020). Moreover, the basic mechanism involved in the phage infection process in complex systems such as milk and dairy products still needs to be clarified (García-Anaya *et al.*, 2020).

Therefore, the objectives of this work (phase 4, section 3.1) are:

i) study the effect of skimming, heat treatment and presence of raw milk microbiota on the *E. coli* and *S. aureus* phage success as biocontrol agents;

ii) acquire more understanding of the main milk component(s) that negatively affect phage lytic ability.

7.2 Experimental Setup

7.2.1 Study design

This study was designed to evaluate the effect of raw milk-associated microorganisms and skimming of cows' milk fat on the lytic activity of previously selected *E. coli* and *S. aureus* phages (section 6.4). This approach was achieved by first testing the efficacy of *E. coli* and *S. aureus* phages to control their target strain(s) in sterilized whole and skimmed milk. Two concentration levels (10^5, 10^6 PFU/ml) were applied according to the phage minimum inhibitory concentration results (section 6.3.1) and a higher phage concentration (10^8 PFU/ml) was selected due to the expected challenges in dairy environment application (Chapter 2). After that, the ideal phage concentration was tested in raw whole and skimmed milk with a sampling period related to cheese manufacture protocol. In addition, the effect of whole and skimmed (either sterilized or raw) milk components on phages adsorption rate were investigated.

7.2.2 Materials & Methods

7.2.2.1 Bacteriophages, host strains and culture conditions

E. coli phage T4, *S. aureus* phage EBHT and *S. aureus* phage K2 and their respective host strains (Table 3.3) were cultured as described in section 3.4. The high-titre phage suspensions were prepared as described by Sambrook and Russell (2011) in section 3.4.1. The phage titre was determined by the overlay plaque assay as described by Sambrook and Russell (2011) in section 3.4.2.

The *E. coli* and *S. aureus* host stains were cultured as described in sections 3.3.1 and 3.3.2, respectively. To prepare broth cultures of host strains for biocontrol activity testing in milk, each bacterial strain was streaked on to a Nutrient agar (NA) plate (LAB M., UK) and incubated at 37 °C for 18 h. A single colony from each NA streak plate was then inoculated into Luria Bertani (LB) Broth (Fisher; US) and incubated at 37 °C for 24 h.

7.2.2.2 Sampling and testing media preparation

Raw cow's milk obtained from HAU bulk tank was transferred to the laboratory within 10 min and the pH value and temperature were measured. Skimmed milk was prepared by using cream separator (Milky FJ 90 PP, Austria). The fat percentage of the whole and skimmed milk was analysed by MilkoScan (MilkoScan^{TM} Minor 78110, FOSS, Denmark) in duplicate. Raw whole and skimmed milk were kept at 4 °C prior to their use within 1 – 4 h.

Sterilized milk was prepared by autoclaving at 121 °C for 10 min and a standard plate count using NA (LAB M., UK) medium was used to check sterilization efficiency.

7.2.2.3 Challenge tests in sterilized and raw cows' milk

Firstly, three concentrations (10^5, 10^6 and 10^8 PFU/ml) of *E. coli* phage T4 and *S. aureus* phage cocktail (1:1 EBHT and K2) were tested separately against 10^4 CFU/ml of respective host strain in sterilized whole and skimmed milk. *E. coli* or *S. aureus* count and phage titre were determined at zero time and after 2, 4, 6 and 24 h. Milk-plus-host and milk-plus-phage controls were also included.

Secondly, 10^8 PFU/ml of *E. coli* phage T4 and *S. aureus* phage cocktail were tested separately against 10^4 CFU/ml host strain in raw whole and skimmed milk. *E. coli* or *S. aureus* count was determined at zero time and after 30, 60, 90, 120 min and 24 h. Milk only, milk-plus-host and milk-plus-phage controls were also included.

All treatments were performed in duplicate and incubated at 37 °C with shaking at 100 rpm. Collected samples were centrifuged for 10 min at 5800 rpm (2-16PK, Sigma, Germany) and the supernatants were tested by overlay plaque assay to determine phage count as described in section 3.4.2. *E. coli* and *S. aureus* counts were determined on the pellet by plating decimal dilutions on selective Tryptone Bile Glucuronide Agar (TBX) media and Baird-Parker agar medium with 5 % egg yolk tellurite (LABM, UK), respectively. *E. coli* blue colonies and *S. aureus* black colonies with opaque halos only were counted after incubation at 37 °C for 24 h.

7.2.2.4 Determination of phage adsorption rate

E. coli phage T4 and *S. aureus* phage EBHT adsorption rate was conducted as described by Agún *et al*. (2018) in duplicate. Overnight cultures of host strains were 10-fold serially diluted in sterilized (whole and skimmed) and raw (whole and skimmed) milk, then 900 µl of each diluted milk (10^8 CFU/ml) were mixed with 100 µl of a phage stock containing 10^8 PFU/ml (multiplicity of infection (MOI) = 1).

A control sample was prepared by mixing 900 µl of SM buffer with 100 µl of phage stock. All samples were incubated for 20 min at 37 °C to allow phage adsorption. After that, samples were centrifuged for 10 min at 5800 rpm at 4 °C and the supernatants were separated and phage titre was determined by overlay plaque assay to determine the number of non-adsorbed phages.

The phage adsorption rate % was calculated as follow:

Phage adsorption rate % = ((phage number $_{\text{in control supernatant}}$ − phage number $_{\text{in sample supernatant}}$) / (phage number $_{\text{in control supernatant}}$)) × 100.

7.2.3 Statistical analysis

Milk treatments were performed in duplicate and dilutions were plated in duplicate at each time interval. For all treatments, the bacterial count (*E. coli* and *S. aureus*) and phage titre were expressed as log CFU/ml and PFU/ml, respectively. Results are presented as logarithmic means ± standard deviation (n = 4). A general design analysis of variance (ANOVA) was carried out to determine any significant differences ($p \leq 0.05$) among the treatments and comparison between means by Duncan's multiple range test ($p \leq 0.05$) using GenStat software (GenStat V 18.1. 17005, England, UK).

7.3 Results & Discussion

7.3.1 Phage biocontrol activity in sterilized milk

E. coli phage T4 and *S. aureus* phage cocktail were initially tested at three concentrations (10^5, 10^6 and 10^8 PFU/ml) against host strain (10^4 CFU/ml) in sterilized whole and skimmed milk after 2, 4, 6 and 24 h of incubation at 37 °C.

The *E. coli* phage T4 efficacy to control *E. coli* (NCIMB 10243) growth in sterilized whole and skimmed milk, is shown in Figures 7.1 and 7.2, respectively. The *E. coli* count were increased gradually to 8.5 ± 0.3 and 8.2 ± 0.2 log CFU/ml in sterilized whole and skimmed milk, respectively, after 24 h in absence of the three phage T4 concentrations. After 2 h, 10^5 PFU/ml phage was able to decrease ($p \leq 0.05$) *E. coli* count to log 2 CFU/ml, however, after that *E. coli* phage lost control and the initial *E. coli* count increased to log 7.9 after 24 h of incubation in all tested samples.

In addition, the achieved results showed that using 10^8 PFU/ml phage concentration was able to decrease (p= 0.005) the initial *E. coli* count to undetectable levels (≤ 10 CFU/ml) after 2 h of incubation in sterilized whole and skimmed milk, and after 6 h using 10^6 PFU/ml phage. In particular, phage T4 treatments data using the three levels of phage concentration demonstrated no significant ($p \leq 0.05$) differences when tested in sterilized whole and skimmed milk.

The behaviour of phage titres in both sterilized milks was similar at the three phage concentration levels. Particularly, phage titre showed an average increase after 24 h to 7.6 ± 0.2 log PFU/ml at 10^5 and 10^6 PFU/ml phage treatments, and to 8.8 ± 0.1 log PFU/ml at 10^8 PFU/ml phage treatment. Moreover, in absence of host (phage control) the phage titre was stable and decreased (p= 0.292) by only 0.5 ± 0.2 log PFU/ml after 24 h of incubation in all controls.

Many authors argued that milk components interfere with the interaction between phage and target strain (O'Flaherty *et al.*, 2005a; Gill *et al.*, 2006; Guenther *et al.*, 2009; García-Anaya *et al.*, 2020). Furthermore, García-Anaya *et al.* (2019) reported that phages are not homogenously distributed in milk. That lead to reduction of the phage lytic ability in milk or dairy environment. Further

explanation on the effect of milk components on phage biocontrol activity is discussed in section 7.3.3.

These findings are in accordance with Tomat *et al.* (2018) who reported complete inactivation of *E. coli* DH5α (10^3 CFU/ml) after 2 h in RSM using six phage *E. coli* cocktail (10^9 PFU/ml) at 37 °C. Similar observations were reported by Endersen *et al.* (2013), who tested mixture of six mycobacteriophages (10^9 PFU/ml) to control *Mycobacterium smegmatis* (10^3 CFU/ml) in both BHI broth medium and RSM. Endersen found no significant differences between the ability of mycobacteriophages to control host strain in BHI broth and RSM. However, a study by Huang *et al*. (2018) reported that a higher *Salmonella* phage LPST10 concentration (10^5 PFU/ml) was required to inhibit *Salmonella* Typhimurium strains (10^3 PFU/ml) in reconstituted skimmed milk (RSM), than the concentration required (10^3 PFU/ml) in 2xYT broth. Notably, high phage concentration is required to achieve high biocontrol activity in milk.

On the other hand, the stability of phage titre during incubation in dairy environment was previously reported in chocolate milk (Guenther *et al.*, 2012), skimmed milk (Huang *et al.*, 2018; Tomat *et al.*, 2018).

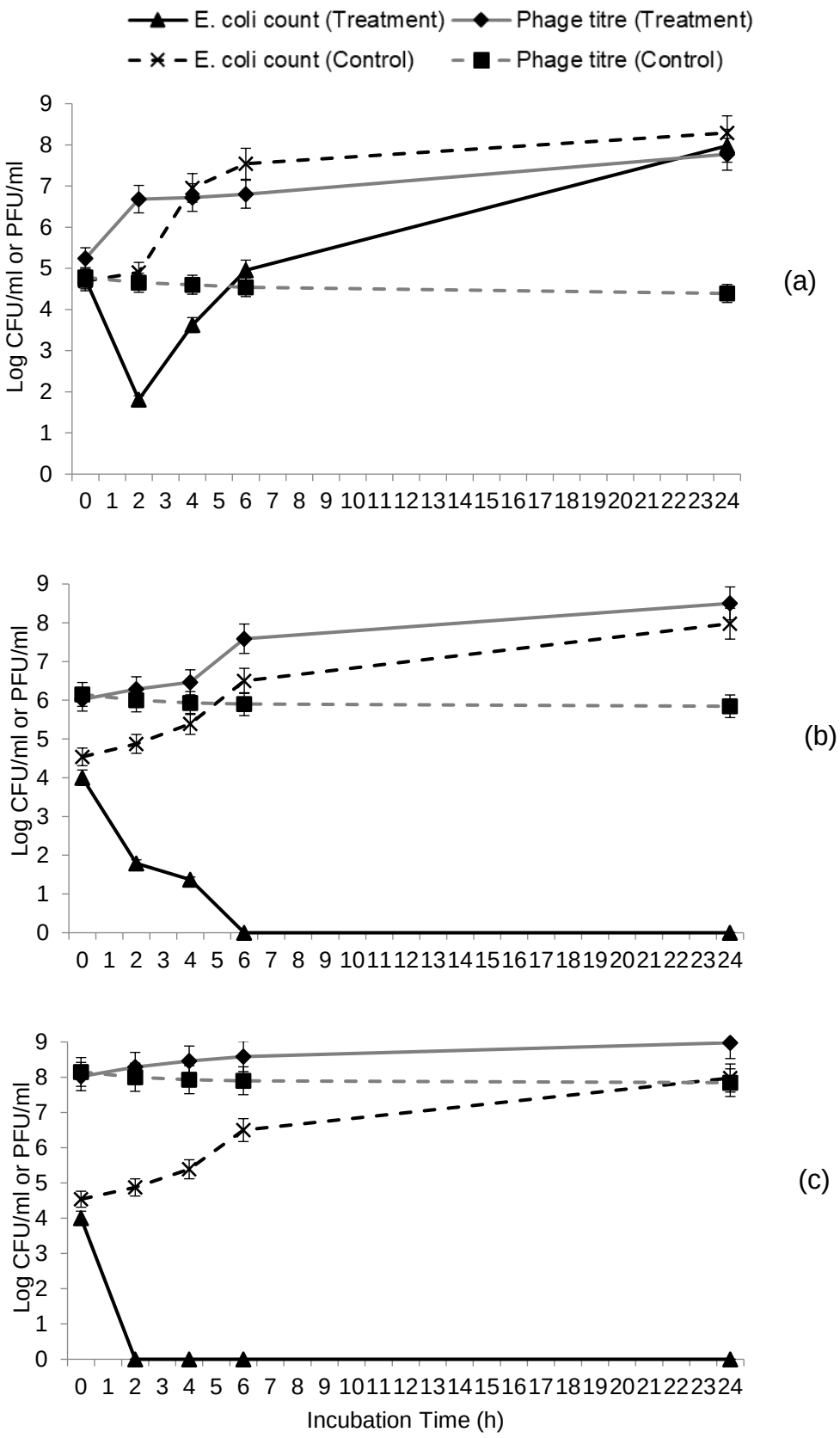

Figure 7.1. Effect of *E. coli* phage T4 on *E. coli* NCIMB 10243 growth in sterilized whole milk using 10^5 (a), 10^6 (b) and 10^8 (c) PFU/ml at 37 °C; Data reported are means ± standard deviations.

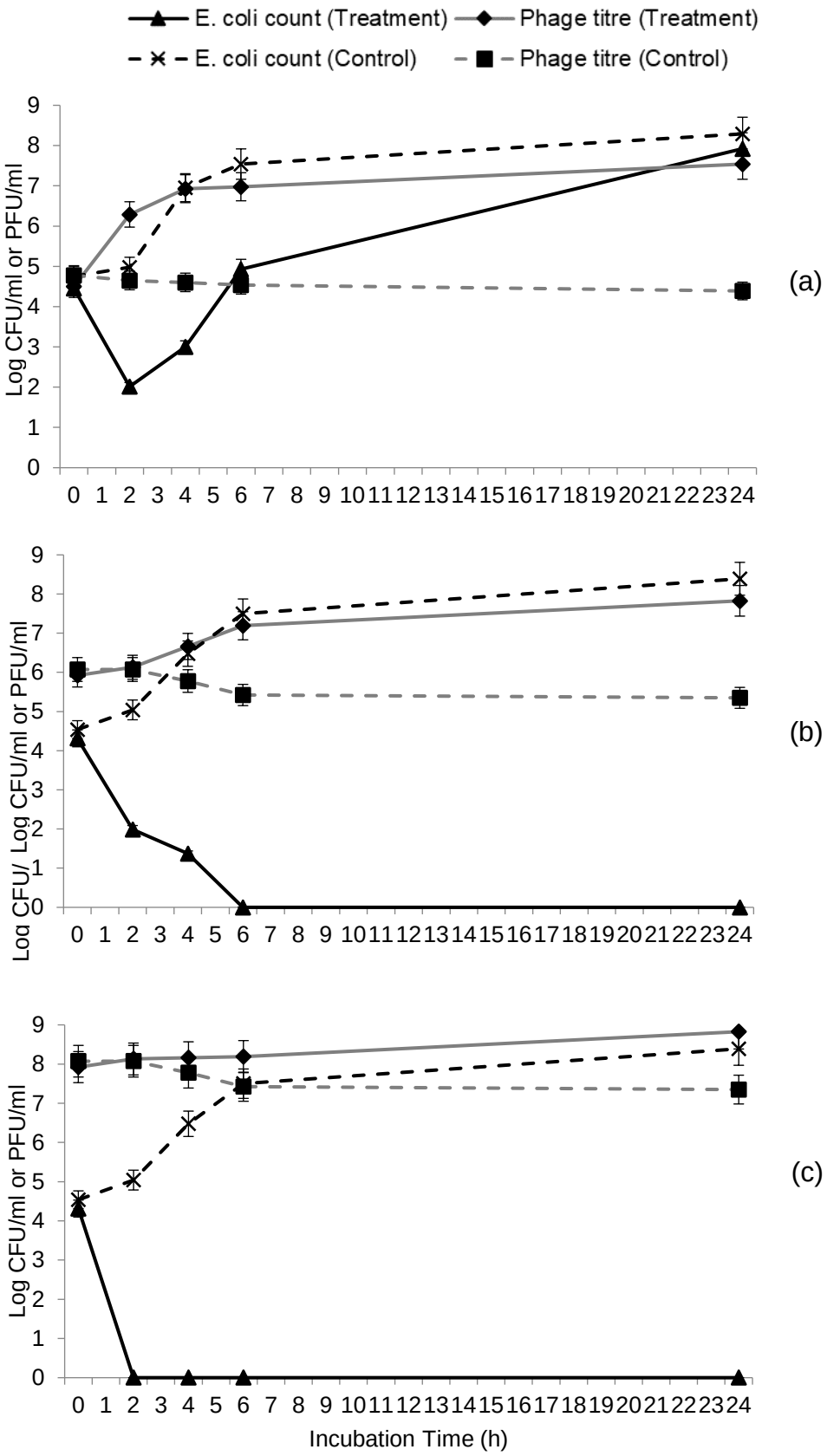

Figure 7.2. Effect of *E. coli* phage T4 on *E. coli* NCIMB 10243 growth in sterilized skimmed milk using 10^5 (a), 10^6 (b) and 10^8 (c) PFU/ml at 37 °C; Data reported are means ± standard deviations.

The *S. aureus* phage cocktail efficacy at 10^5, 10^6 and 10^8 PFU/ml to control the growth of *S. aureus* (DSM 104437 and ATCC 19685) at 10^4 CFU/ml in sterilized whole and skimmed milk, is shown in Figures 7.3 and 7.4, respectively.

The *S. aureus* count were increased gradually to 7.8 ± 0.2 and 7.6 ± 0.1 log CFU/ml in sterilized whole and skimmed milk, respectively, after 24 h in absence of the three phage cocktail concentrations. A similar pattern to *E. coli* phage was recorded, as after 24 h 10^5 PFU/ml phage wasn't able to control *S. aureus* and the count increased to log 7.0 CFU/ml. Likewise, in both sterilized milk types, the 10^8 PFU/ml phage was able to reduce the initial *S. aureus* contamination below the detection limit (≤ 10 CFU/ml) after 2 h, but only after 24 h using 10^6 PFU/ml phage. Of note, similar to *E. coli* phage data, collected results from *S. aureus* phage cocktail treatments at the three concentration levels demonstrated no significant ($p \leq 0.05$) differences when tested in sterilized whole and skimmed milk.

The behaviour of *S. aureus* phage cocktail titres in both sterilized milks was similar at the three phage concentration levels. Particularly, phage titre showed an average increase after 24 h to 8.0 ± 0.3 log PFU/ml at 10^5 and 10^6 PFU/ml phage treatments, and to 9.8 ± 0.1 log PFU/ml at 10^8 PFU/ml phage treatment. Moreover, in absence of host (phage control) the phage titre was stable and decreased ($p \leq 0.05$) by 0.6 ± 0.2 log PFU/ml after 24 h of incubation in all controls.

These results are in concurrence with García *et al.* (2007) who found that *S. aureus* phage mixture (Φ35 and Φ88) at 10^6 and 10^8 PFU/ml phage concentration was able to inhibit the growth of *S. aureus* Sa9 (10^6 CFU/ml) in LB medium after 8 h of incubation at 37 °C. However, higher phage titre was required (10^8 PFU/ml) for complete cell inactivation after 2 h until the end of 8 h of incubation in commercial UHT whole milk.

These data demonstrate that tested phages had a high potency to eliminate or suppress any further growth or contamination of *E. coli* and *S. aureus* pathogens after 24 h. Moreover, the ability to detect the phage titre in milk revealed that milk components could interfere with lytic ability instead of phage

viability. In contrast, García-Anaya *et al.* (2019) found that around 50 % of inoculated phages were not able to form plaques when applied in raw whole milk.

Interestingly, these data showed that *E. coli* phage T4 and *S. aureus* phage cocktail had similar biocontrol activity either applied in sterilized whole or skimmed milk, and to the extent of our knowledge this is the first study comparing unhomogenized heat treated whole and skimmed milk. In relative approach, Zinno *et al.* (2014) recorded that *Salmonella* phage P22 (10^{12} PFU/ml) was able to eliminate *Salmonella* Typhimurium LT2 (10^{4} CFU/ml) with no significant ($p \leq 0.01$) differences between commercial pasteurized whole and skimmed milk application after 48 h of incubation at 4 °C. The phage treatment in raw milk (section 7.3.2) and phage adsorption rate to milk components (section 7.3.3) results gave further clarification of this approach.

As a conclusion, 10^{8} PFU/ml phage concentration showed the highest biocontrol activity in sterilized whole and skimmed milk, therefore this concentration was selected to be applied in raw whole and skimmed milk (section 7.3.2).

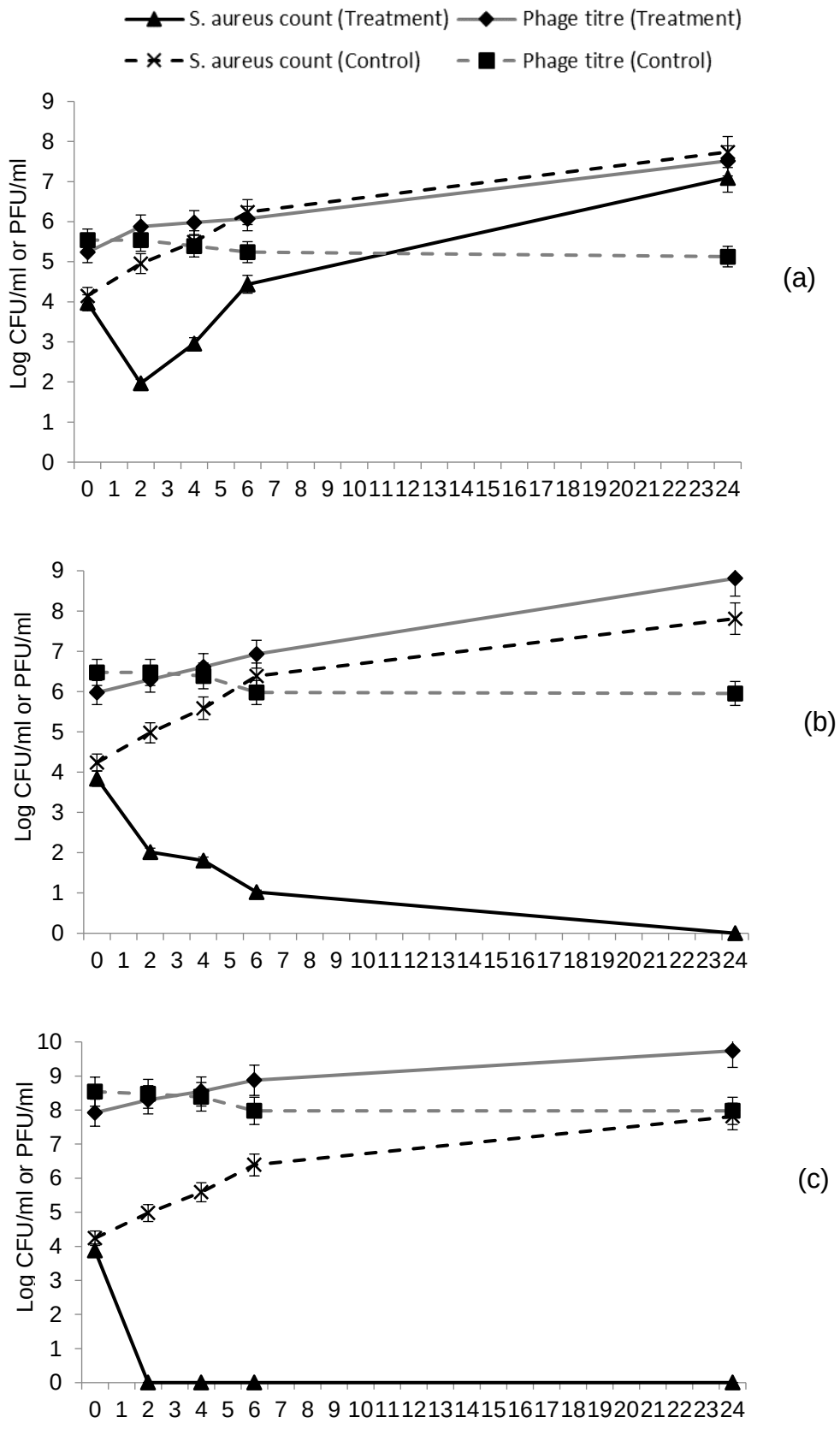

Figure 7.3. Effect of *S. aureus* phage cocktail on *S. aureus* (DSM 104437 and ATCC 19685) growth in sterilized whole milk using 10^5 (a), 10^6 (b) and 10^8 (c) PFU/ml at 37 °C; Data reported are means ± standard deviations.

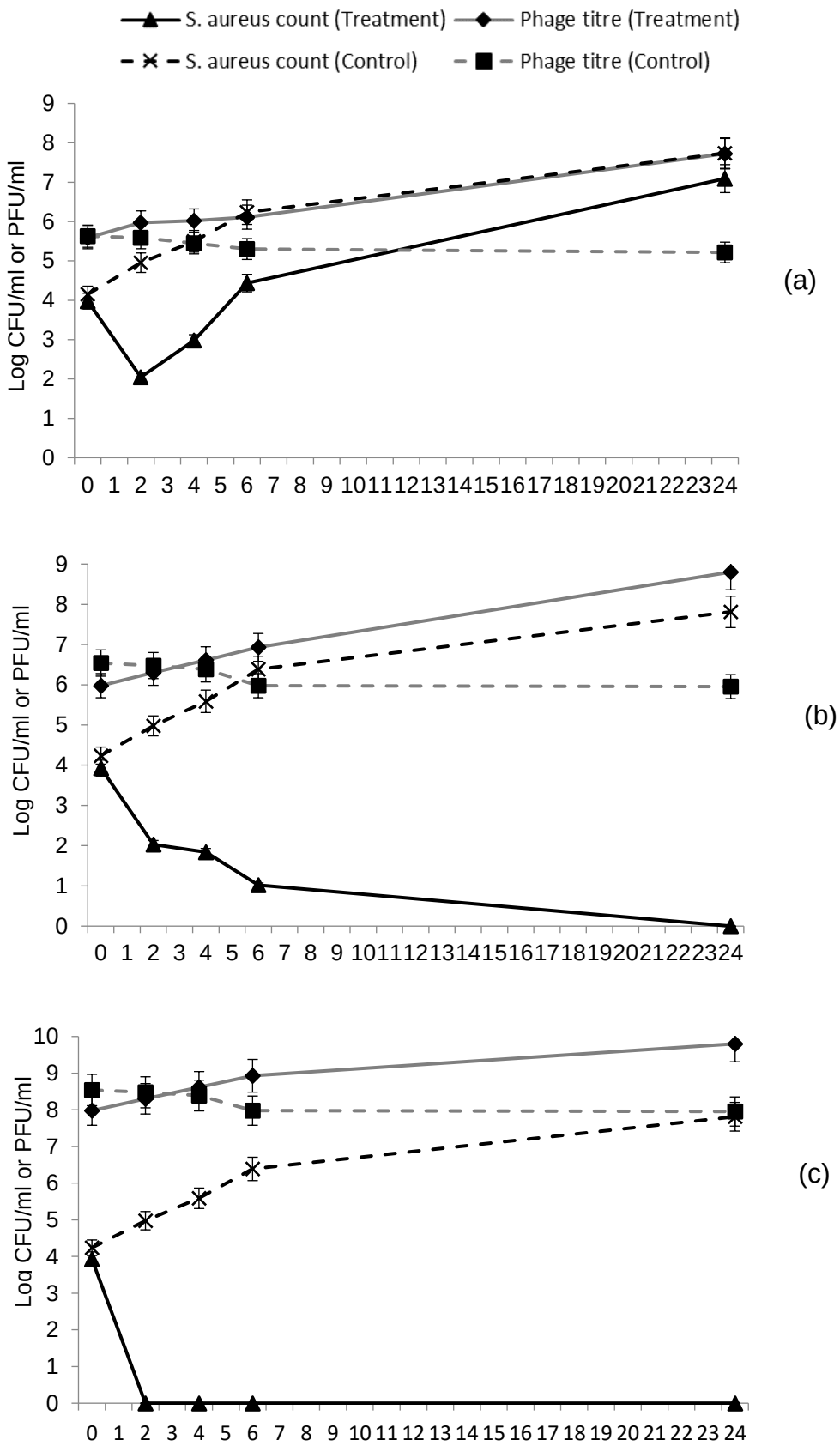

Figure 7.4. Effect of *S. aureus* phage cocktail on *S. aureus* (DSM 104437 and ATCC 19685) growth in sterilized skimmed milk using 10^5 (a), 10^6 (b) and 10^8 (c) PFU/ml at 37 °C; Data reported are means ± standard deviations.

7.3.2 Phage biocontrol activity in raw milk

E. coli phage T4 and *S. aureus* phage cocktail were tested using 10^8 PFU/ml phage concentration against respective host strain (10^4 CFU/ml) in raw whole and skimmed milk after 30, 60, 90, 120 min and 24 h of incubation at 37 °C (Figure 7.5).

An average *E. coli* decrease of 2.0 log CFU/ml was recorded after 2 h in both raw whole and skimmed milk. However, after 24 h phage T4 showed higher ($p \leq 0.05$) ability to control *E. coli* growth in raw skimmed milk (3.6 log CFU/ml) compared to application in raw whole milk (4.6 log CFU/ml), while *E. coli* strains increased to around 8.2 log CFU/ml in the control (raw milk + host) (Figure 7.5a). The inability of *E. coli* phage T4 to completely suppress the growth of inoculated and naturally occurring *E. coli* could be attributed to the observed narrow host range of phage T4 (section 6.3.3). That may therefore justify the presence of *E. coli* in the treated samples after 24 h of incubation, although, phage T4 was still able to decrease ($p \leq 0.05$) *E. coli* viable count by 4.0 log units compared to untreated samples.

On the other hand, *S. aureus* phage cocktail was able to decrease the *S. aureus* viable count to undetected levels (≤ 10 CFU/ml) after 24 h of incubation in both raw whole and skimmed milk (Figure 7.5b). Due to the broad host range of phage EBHT and K2 (section 6.3.3), the phage cocktail was able to eliminate both inoculated and naturally occurring *S. aureus*, while in the absence of phage the *S. aureus* population increase to log 7 CFU/ml.

The recorded decrease in phage biocontrol activity in raw milk is in correspondence with Porter *et al.* (2016) who found that *E. coli* phage cocktail (10^8 PFU/ml) was able to reduce *E. coli* P5-AmpR (10^4 CFU/ml) viable count below 10 CFU/ml after 1 h of incubation and a high increase in count to around 10^3 CFU/ml after 2 h and around 10^4 CFU/ml was still detected after 12 h, however a complete host elimination was achieved in liquid media after 1 h. One major criticism of this study, that *E. coli* count in raw milk was based on plating on TSA plates as a total bacterial count with using raw milk that had TBC of ≤ 100 CFU/ml, where the effect of phage treatment on natural or inoculated *E. coli* was not identified.

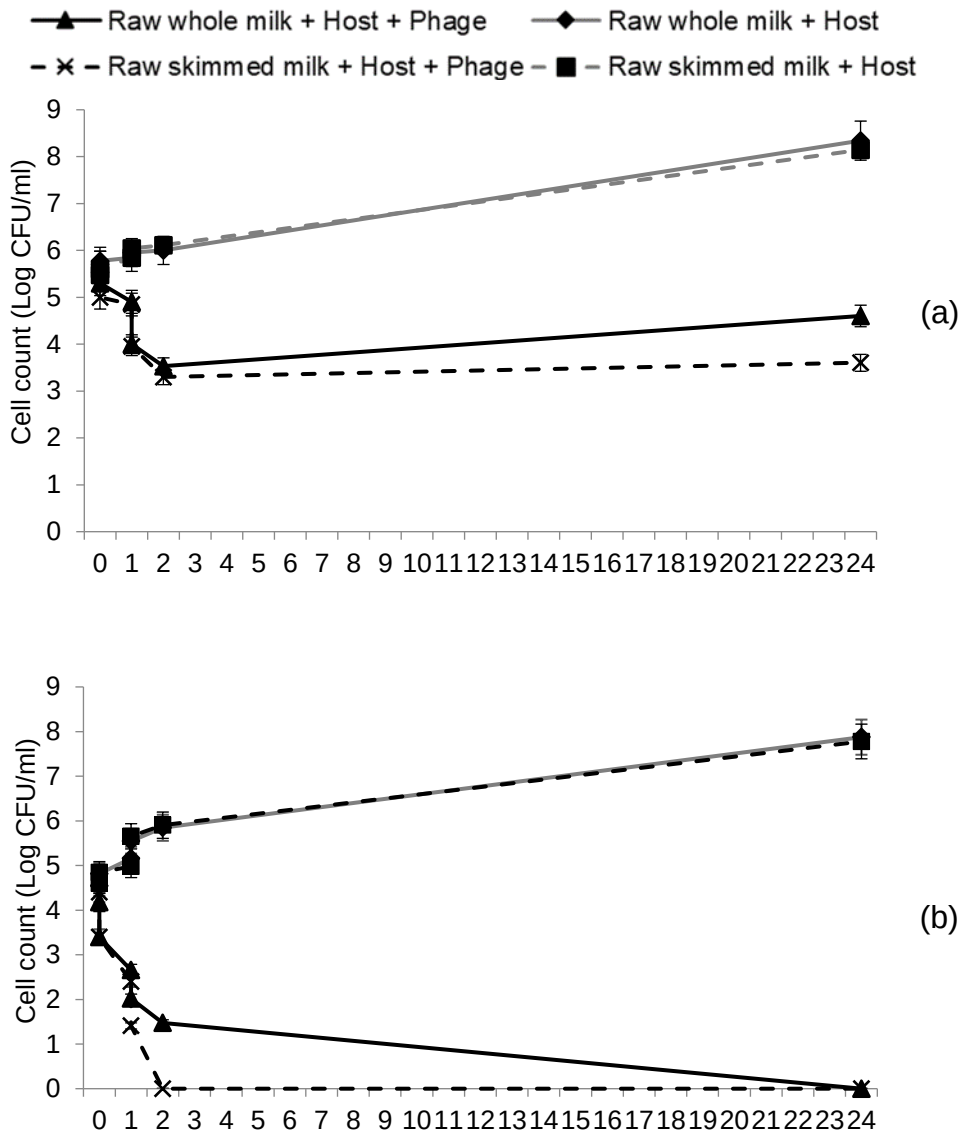

Figure 7.5. Effect of *E. coli* phage T4 (a) and *S. aureus* phage cocktail (b) on inoculated and naturally occurring *E. coli* or *S. aureus* growth in raw milk using 10^8 PFU/ml at 37 °C; Data reported are means ± standard deviations.

Furthermore, O'Flaherty *et al.* (2005a) found that *S. aureus* phage K (10^9 PFU/ml) was able to inhibit *S. aureus* DPC5645 (10^7 CFU/ml) within 2 h of inoculation at 37 °C in BHI and heat-treated whole milk (90 °C for 10 min), when the host strain was inoculated 4 h in advance, while no detected reduction in the cell count occurred when applied in raw whole milk. In addition, García *et al.* (2009) demonstrated that after *S. aureus* phage mixture treatment, the *S. aureus* cell count was lower (log 3.7 CFU/ml) significantly ($p \leq 0.05$) in raw semi-skimmed milk than achieved in raw whole milk (log 4.8 CFU/ml) compared to untreated samples (log 6.0 CFU/ml); where the *S. aureus* reduction has observed earlier in raw semi-skimmed milk (at zero time) compared to raw whole milk (after 6 h). The lower phage biocontrol activity in raw milk compared by heat treated milk is also reported by McLean, Dunn and Palombo (2013) and Hu, Meng and Liu (2016).

The use of several phages which target different strains within the same species showed to be effective in controlling *S. aureus* in raw milk. The use of phage cocktails has been reported for control of various foodborne pathogens in dairy application such as, *E. coli* (Porter *et al*., 2016; Tomat *et al*., 2018), *S. aureus* (García *et al.*, 2009), *L. monocytogenes* (Guenther *et al.*, 2009), *Pseudomonas* spp. (Hu, Meng and Liu, 2016) and *Mycobacterium smegmatis* (Endersen *et al.*, 2013). According to these studies, using a mixture of phages enhances the effectiveness of phage treatment for biological control compared to single phage application. Whilst, after 2 h variable results were noticed. As, similar to phage cocktail data in sterilized milk, the phage cocktail was able to eliminate *S. aureus* in raw skimmed milk, however in raw whole milk *S. aureus* decreased initially to 1.5 log CFU/ml.

Although this is the first study focusing on phage biocontrol activity in raw milk with different fat concentration. Both control and treated samples showed noticeably higher *S. aureus* count compared to raw milk samples. That may relate to the high competition between inoculated pathogen and natural microbiota (McLean, Dunn and Palombo, 2013), which subsequently affected significantly the biocontrol activity results.

Achieved results, variability and limitations stated of previous studies using phages as biocontrol agent in dairy environment, emphasis the necessity

of studying the influence of milk components on the phage lytic ability. Therefore, to further understand the behaviour of phage in milk, the phage adsorption rate to milk components was performed.

7.3.3 Effect of skimming of cow's milk and raw milk-associated microorganisms on phage lytic activity

E. coli phage T4 and *S. aureus* phage EBHT adsorption rate percentages in sterilized (whole and skimmed) and raw (whole and skimmed) milk are shown in Figure 7.6. The highest (p= 0.755) adsorption rate were found in raw whole milk (87.8 %) followed by sterilized whole milk (57.7 %), while, sterilized skimmed milk (37.6 %) resulted in a higher ($p \leq 0.05$) adsorption rate than average percentages in raw skimmed milk (31.7 %).

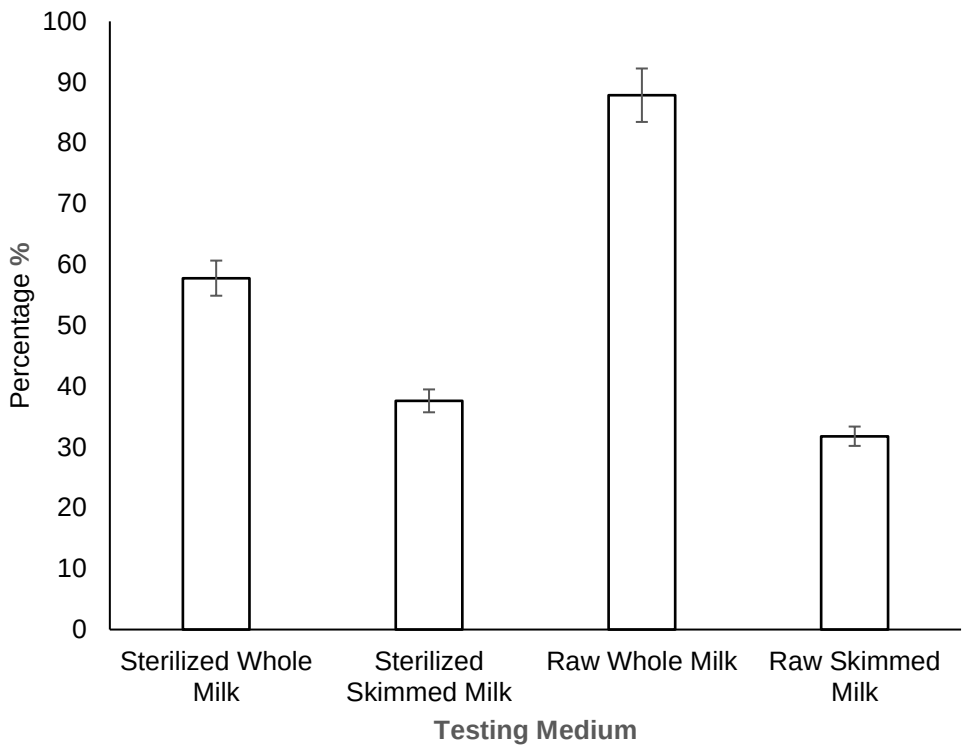

Figure 7.6. The average adsorption rate percentages of *E. coli* phage T4 and *S. aureus* phage EBHT on sterilized (whole and skimmed) and raw (whole and skimmed) milk; Data reported are means ± standard deviations.

As, phage infection begins with adsorption of phage particles to specific receptor sites on the host cell surface (Fister *et al.*, 2016a), any interference with that interaction will subsequently inhibit phage attachment and thereby negatively affect the phage lytic ability (Gill *et al.*, 2006).

According to previous collected results and phage biocontrol activity results in sterilized whole and raw milk, the factors that affect biocontrol activity could be hypothesised as follows. The first factor is the presence of non-target components which decrease the probability for contact between phage and target bacteria. Naturally, milk consists of fat, protein (casein), whey proteins and lactose, in addition to other constituents such as somatic cells (white blood corpuscles or leucocytes), which range between ≤ 200 000 cells/ml up to 400 000 cells/ml in healthy animal (Soboleva, 2013; Ndahetuye *et al.*, 2020).

Thus, this may justify the decrease in raw whole and skimmed milk biocontrol activity, even when phage adsorption to sterilized skimmed milk was higher than raw skimmed milk. However, the other milk constituents (somatic cells and impurities) are decreased at milk clarification, which is an essential step prior to milk pasteurization and homogenisation (Fox, 2003). This could explain the variability in previous studies on commercial and small-scale pasteurized milk depending on applied treatments to the tested milk.

The second factor, the adsorption of target strain to milk components (mainly proteins and fat), also may prevent the attachment between phage and target strain. D'Incecco *et al.* (2018) concluded that milk immunoglobulins (Ig), especially IgA and IgM combined to *Clostridium tyrobutyricum* cell surface causing aggregation between bacterial cells and milk fat globules. The authors argued that this process is enhanced by the presence of antigens on the bacterial cell surface that play the role of ligands for immunoglobulins.

Gill *et al*. (2006) tested the degree of binding of *S. aureus* phage K (ATCC 19685, the same used in this study) (10^{10} PFU/ml) in filter sterilized and heat treated (100 °C for 10 min) raw skimmed milk whey (collected from 23 different cows) to *S. aureus* strain Newbould 305 (10^{8} CFU/ml). The phage binding assay and fluorescence microscope imaging revealed that raw whey decreased the phage binding to the *S. aureus* cells, by accumulation of whey protein on the bacterial cell surface, and this was not detected in heat treated samples. However, it was noticed that this effect was highly variable within individual cows, as phage binding in four whey samples showed no significance differences compared to testing in TSB medium.

O'Flaherty *et al.* (2005a) added that in raw whole milk live *S. aureus* DPC5645 cells clumped together and low phage K lytic ability was noticed, while, in heat-treated whole milk no clumping of live cells was observed and phage K gave higher biocontrol activity. However, they recorded higher phage lytic ability in raw and heat-treated whey compared to homogenized and low heat-treated milk (45 °C for 10 min). As well, there was no significant differences between the tested phage lytic ability in sterilized whole and skimmed milk (section 7.3.1), while differences were noticeable between raw whole and skimmed milk. This shows that phage lytic ability is more susceptible to heat-labile components related to milk fat instead of whey proteins.

The third factor, the adsorption of phage to milk components, that prevents the attachment between phage and target strain. In the same approach, Yan *et al.* (2021) tested *S. aureus* phage endolysin (LysGH15) in whole milk, extracted fat solution and extracted lactose solution. They observed that lactose had relatively low (16 %) effect on lytic activity compared to fat (94 %). This supports García-Anaya *et al.* (2019) findings who studied the influence of raw milk homogenization on phage affinity to milk components using *L. monocytogenes* phage A511 (10^8 PFU/ml). They demostrated that tested phage in raw milk showed high ($p \leq 0.05$) affinity to milk fat globules followed by casein, while the lowest ($p \leq 0.05$) adsorption was recorded in whey. However, after raw milk homogenization milk fat loses its ability to bind phages, and higher affinity was observed for casein rather than whey.

To date, the exact component that have the high influence on phage lytic ability in raw milk is unknown. From the findings above, it could hypothesize that immunoglobulins, precisely IgM and IgA, are the main factor that interferes with phage-bacteria interaction and consequently reduce or suppress phage antibacterial activity. Gill *et al.* (2006) reached the conclusion after raw whey fraction and chromatography that IgG is not responsible for the decrease in phage lytic ability, because of the low recorded concentration, whilst an integrative approach in recent studies (Hansen, Larsen and Wiking, 2021; Wiking *et al.*, 2022) found that IgG is less associated to milk fat globules and not involved in agglutination process. However, IgM and IgA are the only polymeric immunoglobulins that can form dimers, are highly associated with milk fat

globules and their main role is in agglutination mechanism (D'Incecco *et al.*, 2018).

Milk immunoglobulins can be inactivated (partially or completely) during milk manufacturing process of homogenization and pasteurization (Mulder and Walstra, 1974) which could explain the achieved results and García-Anaya *et al.* (2019) findings. Mainer *et al.* (1997) evidenced that Low Temperature-Long Time (LTLT) processing (63 °C for 30 min) led to only 33 % denaturation of milk IgM and no recorded effect on other Ig's, while the standard High Temperature-Short Time (HTST) process (72 °C for 15 sec) showed denaturation of 1 % IgG, 2 % IgA and 14 % IgM. Moreover, all immunoglobulins would be denatured by milk ultra-heat treatment (UHT) and sterilization (Kummer *et al.*, 1992). Following achieved hypothesis, this could explain the variability in phage biocontrol activity in previous studies, when tested in heat treated milk.

7.4 Conclusions

E. coli phage T4 and *S. aureus* phage cocktail were successful in eliminating target strains in sterilized whole and skimmed milk. Moreover, *S. aureus* phage cocktail was able to reduce ($p \leq 0.05$) the initial *S. aureus* contamination below the detection limit (≤ 10 CFU/ml) in raw whole and skimmed milk, however due to narrow *E. coli* phage T4 host range around 4 log CFU/ml were still detected after 24 h of incubation. The tested phages at 10^8 PFU/ml concentration showed high antimicrobial activity, showcasing their high potential to improve microbial safety of raw milk cheeses such as Karish, Domiati and Ras (Chapter 8).

Achieved results gave a better understanding of the behaviour of phages as biocontrol agent in raw milk, which is required to develop new strategies to control pathogens in artisanal cheeses. In order to achieve high phage efficacy in general dairy application, several measures were observed to be necessary for efficient application: a) it is recommended to apply a mixture of phages with wide host range; b) phage treatment should be started 2 h prior to technological treatment such as cheese manufacture and packaging to get the highest phage efficacy; c) increase the time and temperature for raw milk pasteurization intended to be used in phage application.

Overall, phage-based reduction of *E. coli* and *S. aureus* was possible within a challenging high contamination level and non-target microbial population, which could open new approaches of phage application in different products made from raw milk. The reached hypotheses of the effect of IgM, IgA and milk fat on the phage lytic ability could create a stronger platform for future studies which are still required.

Chapter Eight: Effectiveness of *E. coli* and *S. aureus* Bacteriophages as Biocontrol Agents in Egyptian Raw Milk Cheeses during Production and Storage

8.1 Introduction

In Chapter 6 previously mentioned phages showed high lytic activity and ability to inhibit target strains at varied physicochemical conditions (temperature, pH and salt), but in practice, all the conditions will be present together at varied levels, which could positively or negatively influence the phage activity in milk (Ly-Chatain, 2014). In addition, the observed restricted host range of *E. coli* phage T4 and *S. aureus* phage (EBHT and K2) when tested against different LAB species (isolated from Egyptian dairy products), confirmed that these phages are ideal candidates to be applied without interfering with fermentation and ripening process (section 6.3.3).

In addition, Chapter 7 explored the impact of milk composition and raw milk microbiota on selected *E. coli* phage T4 and *S. aureus* phage cocktail to better understand phage behaviour in a dairy environment. While it was shown in Chapter 7 that the applied phages, in particular *S. aureus* phage cocktail were able to completely eliminate host strains in milk and significantly reduce *Staphylococcus* spp. to undetectable levels, these treatments were conducted in more favorable conditions compared to cheese. Despite the higher phage lytic ability in sterilized milk compared with raw milk, using a heat treatment in artisanal cheese manufacture would not be practical as inhibition of critical enzymes, natural LAB, and whey protein denaturation (Fox, Guinee and Cogan, 2000; Wiking *et al.*, 2022; Tabla *et al.*, 2022) would alter the manufacturing

logistics, quality, flavour and rheological properties of artisanal cheeses (Modi *et al.*, 2001; Ismail *et al.*, 2011; Malek *et al.*, 2012).

In recent years, there has been an increasing interest in using bacteriophages as biocontrol agent in cheese products, however most studies have tended to apply phage at the cheese surface or after complete manufacture (Carlton *et al.*, 2005; Guenther and Loessner, 2011; Soni *et al.*, 2012; Perera *et al.*, 2015; Henderson *et al.*, 2019). However, in all cases complete elimination of target pathogen was not achieved, due to the impact of the food matrix on phage accessibility to the target strain (LeLièvre *et al.*, 2019). Few researchers have studied phage treatment in milk before cheese process, in particular using raw milk.

Therefore, the objectives of this work (phase 5, section 3.1) are to independently apply *E. coli* phage T4 and *S. aureus* phage cocktail (10^8 PFU/ml) to control inoculated (10^4 CFU/ml) and naturally present target strain in Karish, Domiati and Ras cheese at the beginning of manufacture (in milk), following the same validated procedures and conditions under small scale laboratory conditions, in order to quantify the efficiency of phages in controlling pathogen growth during cheese manufacture and storage.

8.2 Experimental Setup

8.2.1 Study design

This study was designed to quantify the *in-vivo* efficiency of phages to control pathogen growth during cheese manufacture and storage. This approach was achieved by monitoring the influence of previously selected *E. coli* phage T4 and *S. aureus* phage cocktail (section 6.3.3) as biocontrol agent in Egyptian raw milk cheeses (Karish, Domiati and Ras). The phage concentration (10^8 PFU/ml) that showed the highest lytic ability in sterilized and raw milk was applied in all cheese treatments.

Given that the highest lytic ability of *E. coli* phage T4 and *S. aureus* phage cocktail was evidenced at the first two hours of inoculation, the phage treatment in Karish, Domiati and Ras cheeses was designed to be applied 1 h prior to the start of curdling process. This would enable the inoculated phage to target respective pathogen for around 1.5 h prior to changing of milk matrix from liquid state to gel (solid) state, without altering the balance between acid development and curd formation.

8.2.2 Materials & Methods

8.2.2.1 Bacteriophages, host strains and growth conditions

A single colony from obtained plates as described in sections 3.3.1 and 3.3.2 for *E. coli* (NCIMB 10243) and *S. aureus* (DSM 104437 and ATCC 19685) host strains, respectively, was streaked on to a Nutrient agar (NA) plate (LAB M., UK) and incubated at 37 °C for 18 h. A single colony from the NA plate was subsequently inoculated in 10 ml LB broth (Fisher; US) and grown aerobically at 37 °C with shaking (200 rpm) to an OD_{600} of 0.9 to 1.0. *E. coli* phage T4, *S. aureus* phage EBHT and *S. aureus* phage K2 and their respective host strains (Table 3.3) were cultured separately as described in section 3.4. The phage stock was prepared as described by Sambrook and Russell (2011) in section 3.4.1.

The serial dilution of host strain or phage stock was prepared in sterilized whole or skimmed milk. This process was conducted to obtain the desired host

(10^4 CFU/ml) and phage (10^8 PFU/ml) concentrations without altering raw milk pH and chemical composition used for cheese manufacture.

8.2.2.2 Challenge tests in Karish, Domiati and Ras cheeses

Karish, Domiati and Ras cheeses were manufactured from raw skimmed milk (Karish) and raw whole milk (Domiati, Ras) at small scale in the HAU category two containment laboratory. The raw cow's milk was obtained from the HAU bulk tank and transferred to the laboratory within 10 min. Treatments were conducted over a 5 months period (from May to September 2021) during the same milking season of previous cheese manufacture (Chapter 5) to avoid any compositional and microbiological variations between both treatments, to manufacture cheeses with the same physicochemical conditions as achieved in section 5.3.

The average total bacterial count (TBC) of HAU bulk tank milk was around log 3 to log 4 CFU/ml for skimmed and whole milk, respectively. However, the total bacterial count of Egyptian raw cow's milk ranges between 5 and 8 log CFU/ml (average 7 log CFU/ml) (Ibrahim, Sharaf and El-khalek, 2015; Sobhy, Hassaan and Shedeed, 2016) in winter and summer, respectively (Mahrous, 2014). The collected raw milk was stored at 4 °C for 16 h prior to use to increase the TBC of raw milk to mimic the typical recorded TBC for Egyptian raw milk, and 0.1 % MO 11 starter culture (Composed of: *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*) was also added (Proquiga, Spain). These were critical steps mainly to test the phage efficacy under high non-target microbial content, which gives a complete understanding of phages potential success under conditions similar to the Egyptian artisanal style.

Firstly, raw milk was prepared simultaneously in four independent cheese vats and the temperature was adjusted at 37 °C, the phage treatment (T) was performed prior to the start of manufacture process as follows: a) the previously diluted phage and host strain suspension (section 8.2.2.1) was inoculated to achieve 10^8 PFU/ml and 10^4 CFU/ml concentration, respectively. *S. aureus* phage cocktail (EBHT and K2) and host strain (DSM 104437 and ATCC 19685) were inoculated in 1:1 ratio; b) therewith, the milk was stirred well to ensure efficient phage and host distribution; c) inoculated milk samples were then

collected at zero time and after 30 and 60 min of incubation and immediately analysed. For control purpose, the other vats were processed as follows: cheeses made from raw milk only (C1), raw milk-plus-host (C2) and raw milk-plus-phage (C3).

After that, milks were processed into Karish, Domiati or Ras following the standard procedures as described in sections 3.2.1, 3.2.2 and 3.2.3, respectively. The pH and temperature were measured during cheese manufacture and storage using a portable electrode for cheese analysis (HANNA HI-99165, UK).

8.2.2.3 Sampling and testing media preparation

After complete curdling of each cheese, curd (25 g) and whey samples (50 ml) were aseptically collected in separate 50 ml plastic sterilized cups. Cheese samples (25 g) were also collected at intervals during cold storage of Karish and ripening time of Domiati and Ras cheese (Table 8.1). Soft cheese samples (Karish and Domiati) were collected from the centre of the cheese after mixing. However, hard cheese samples (Ras) were collected from the centre to the edge of each cheese block (inner core) (~30 g) using a sterilized cheese corer, with cheese rind discarded (~5 g) (Figure 8.1). Ras cheese block was sealed by clear cheese coating (GN Ltd, UK) after sampling to avoid any contamination. All samples were kept at 4 °C for no longer than 4 h before analysis.

Curd and cheese (10 g) samples were homogenized in a Stomacher Lab-blender (Type 400; Seward, London, UK) for 8 min with 90 ml of sterile 2 % sodium citrate solution pre-warmed at 45 °C. Whey samples were centrifuged (3-16PK Sigma, Germany) at 5800 rpm, 4 °C for 5 min prior to overlay plaque assay, to remove indigenous microbiota and curd fines that would otherwise affect plaque formation and appearance.

Table 8.1. Sample collection intervals for Karish, Domiati and Ras cheeses during manufacture and storage.

Interval Time		Karish	Domiati	Ras
Zero time		Milk	Milk	Milk
Coagulation time		30 min	30 min	30 min
		60 min	60 min	60 min
		Whey	Whey	Whey
		Curd* 24 h	Curd* 3 h	Curd* 45 min
Cold storage or Ripening time		1 Day**	1 Day	1 Day
		5 Days	7 Days	30 Days
		9 Days	15 Days	60 Days
		15 Days	30 Days	90 Days

*The stage of complete curdling after 24 h of starter addition for Karish or after 3 h and 45 min of rennet addition during Domiati and Ras manufacture, respectively.

**Day at which the cheese manufacture process was completed and kept for storage or ripening.

Figure 8.1. Ras cheese sampling technique used throughout ripening time.

8.2.2.4 Microbiological analysis

Milk, curd and cheese samples from all treatments were serially diluted (when required) in sterile 2 % sodium citrate solution. Total bacterial count was enumerated on Nutrient agar plate (LAB M., UK). Total inoculated and indigenous *E. coli* (blue colonies) were counted on selective Tryptone Bile Glucuronide Agar (TBX) media. Baird-Parker agar medium with 5 % egg yolk tellurite (LABM, UK) was used to differentiate and quantify inoculated and indigenous *S. aureus* (black colonies with opaque halos) and indigenous *Staphylococcus* spp. (black colonies). All plates were incubated at 37 °C for 24 h.

To determine phage titre, milk, whey, curd and cheese samples from treatment (T) and raw milk-plus-phage (C3) were serially diluted in SM buffer and plated using the overlay plaque assay as described by Sambrook and Russell (2011) in section 3.4.2.

8.2.2.5 Bacteriophage retention rate in curd

In order to test the effect of performed technological treatments for each cheese type (Karish, Domiati or Ras) on *E. coli* phage T4 and *S. aureus* phage cocktail retention in curd, the phage retention rate was calculated. The average phage titre in whey (T and C3) for each cheese type, whether treated by *E. coli* or *S. aureus* phage were compared to the inoculated phage titre in milk using the following formula:

$$Phage\ retention\ rate\ \% = \frac{(\text{Phage titre in milk} - \text{Phage titre in whey})}{Phage\ titre\ in\ milk} \times 100$$

8.2.3 Statistical analysis

Cheese treatments were performed in triplicate with duplicate sampling at each time interval and duplicate plating of dilutions. For all treatments, the bacterial count (TBC, *E. coli* and *S. aureus*) and phage titre were expressed as log CFU/g and log PFU/g, respectively. Results are presented as logarithmic means ± standard deviation (n = 12). A general design analysis of variance (ANOVA) was carried out to determine any significant differences ($p \leq 0.05$) among the treatments and comparison between means by Duncan's multiple range test ($p \leq 0.05$) using GenStat software (GenStat V 18.1. 17005, England, UK).

8.3 Results & Discussion

8.3.1 Phage biocontrol activity in Karish cheese

The efficacy of *E. coli* phage T4 and *S. aureus* phage cocktail (10^8 PFU/ml) to control host strain (10^4 CFU/ml) was independently tested during Karish coagulation time and up to 15 days of storage at 4 °C. The fermentation process was very similar in all treatments and matched the previously achieved pH values during Karish cheese small scale manufacture (section 5.3.1).

8.3.1.1 *E. coli* biocontrol during Karish manufacture and storage

The *E. coli* phage T4 efficacy to control the growth of inoculated (*E. coli* NCIMB 10243) and naturally present *E. coli* in Karish cheese, is shown in Figure 8.2. Inoculated and indigenous *E. coli* population increased in C2 (raw milk plus-host) from 5.3 log CFU/g in milk to 6.6 log CFU/g in curd, whereas, *E. coli* population decreased in cheese gradually to 5.3 log CFU/g after 15 days. Phage treatment (T) reduced ($p = 0.007$) inoculated and indigenous *E. coli* from 5.6 to 2.2 log CFU/g in curd (after 24 h). The *E. coli* population increased slightly ($p = 0.103$) at the first day of storage (2.4 log CFU/g), and a continuous reduction ($p \leq 0.05$) to 1.3 log CFU/g was achieved at the end of 15 days of storage. A similar observed effect in raw milk-plus-phage control (C3) confirms the ability of the phage to target the naturally present *E. coli*. However, due to phage T4's narrow host range (section 6.3.3), 1.1 log CFU/g was still present in C3 (raw milk plus-phage) compared with C1 (raw milk only; 3.4 log CFU/g).

These data are in agreement with the results obtained for phage application in raw milk (section 7.3.2) and demonstrates the high ability of *E. coli* strains to survive during acid coagulation which dropped pH from 6.6 (milk) to 4.5 (curd) until the end of 15 days (pH= 4.3) of storage. It is noteworthy that comparing phage T4 results in raw skimmed milk (without starter culture) to these data, where phage T4 with starter reduced *E. coli* to 2.2 log CFU/g instead of 3.6 log CFU/ml in phage T4 without starter after 24 h, showed that added starter culture had a noticeable effect on *E. coli* growth in all controls and treatment (T). Accordingly, the decline in *E. coli* count during Karish cheese cold storage, would be much related to the antagonistic effect between phage, starter and pH.

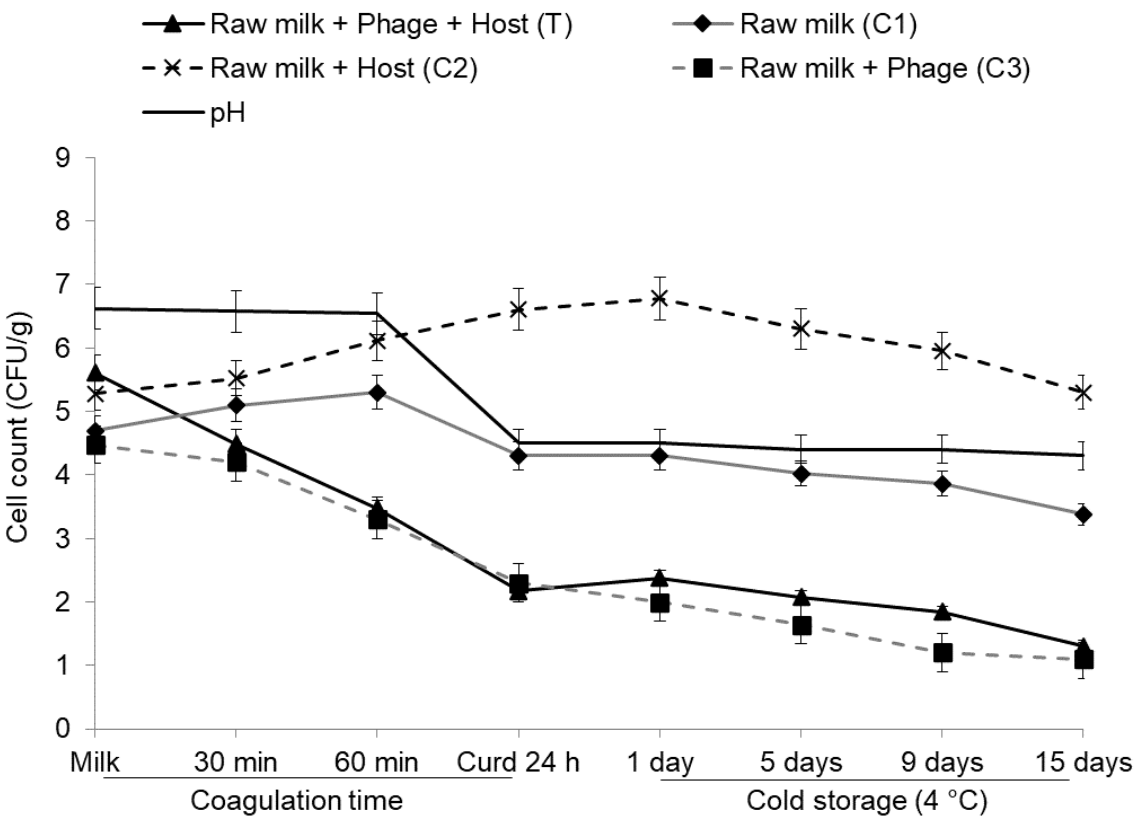

Figure 8.2. Effect of *E. coli* phage T4 (10^8 PFU/ml) on inoculated and indigenous *E. coli* growth during Karish cheese manufacture and cold storage; Data reported are means ± standard deviations of three independent trials with duplicate sampling and measurements.

The ability of *E. coli* to grow or survive the cheese pH was previously reported by several studies. Ibrahim *et al.* (2020) found that *E. coli* O26:H11 was able to survive Karish cheese manufacture reaching to 6.5 and 5.3 log CFU/g after 1 and 15 days of storage, respectively, at 4 °C. Similarly, Ombarak, Saad and Elbagory (2019) showed an increase in *E. coli* O157:H7 after Karish cheese inoculation (10^3 CFU/ml) from around 4.4 to 5.5 log CFU/g after 1 and 8 days of cheese refrigerated storage, respectively. This is linked to the ability of *E. coli* strains to adapt at low (4.5) cheese pH (Farrokh *et al.*, 2013). The authors added that part of the increase of *E. coli* O157:H7 count from milk to curd and cheese is due to the concentration of the bacteria during whey separation, which could explain the small increase in total *E. coli* count at day 1 compared to curd after 24 h.

On the other hand, several studies have attempted to explain the observed effect of added starter culture on reducing *E. coli* growth. Masoud *et al.* (2012) studied the effect of raw milk indigenous microbiota and added starter culture on *E. coli*, *S. aureus* and *L. innocua* survival in raw milk cheese acidified to pH 5.4. They found that *E. coli* count decreased to 5.4 and 4.2 log CFU/g at first day and after 28 days, respectively, in cheese treated by *L. lactis* starter (10^9 CFU/g) and *E. coli* (10^6 CFU/g); these findings are also supported by Vernozy-Rozand *et al.* (2005) and Callon, Arliguie and Montel (2016). In particular, starter culture strains could contribute to hindering foodborne pathogens growth not only by lowering pH but also by production or formation of many compounds such as antimicrobial metabolites (bacteriocins) (Coelho *et al.*, 2014; Aspri *et al.*, 2017), organic acids and hydrogen peroxide (H_2O_2) (Laranjo, Potes and Elias, 2019; García-Díez and Saraiva, 2021).

In addition, the total bacterial count screening during manufacture and storage (Figure 8.3a), showed a high significant ($p = 0.004$) increase from 6.6 (milk) to 9.4 (1 day) log CFU/g in treatment (T) and decreased ($p = 0.693$) gradually to 9.0 log CFU/g at the end of storage. Similar observations were recorded for the three controls. Thus, the total bacterial count increase in T and C3 indicates that phage treatment did not affect the fermentation activity of the added starter culture and the natural milk microbiota during cheese manufacture and storage.

Figure 8.3b, demonstrates that the phage titres in treatment and raw milk-plus-phage control were decreased significantly ($p \leq 0.05$) to 5.2 ± 0.2 log PFU/g in curd, and further reduction (p = 0.704) occurred until the end of 15 days (4.7 ± 0.2 log PFU/g). The decline in phage titre in curd after 24 h could be attributed to the recorded loss of phage in whey (6.6 ± 0.3 log PFU/g) and negative effect of low curd pH on phage survival. Conversely, the stability of phage titre during the cheese cold storage at average pH of 4.4 implies their ability to survive this level of pH and keep the *E. coli* count at low level compared to the phage-free trials.

According to previous studies and achieved data, the starter culture alone was not able to reduce *E. coli* 10^2 and 10^5 CFU/g, whereby they may cause illness within or greater than this concentrations (FDA, 2009). Therefore, *E. coli* phage T4 application showed a good efficiency for keeping generic *E. coli* count at low level over cheese storage period, thence the reduction of toxigenic *E. coli* by using specific phage could be assumed (Peng *et al.*, 2013b).

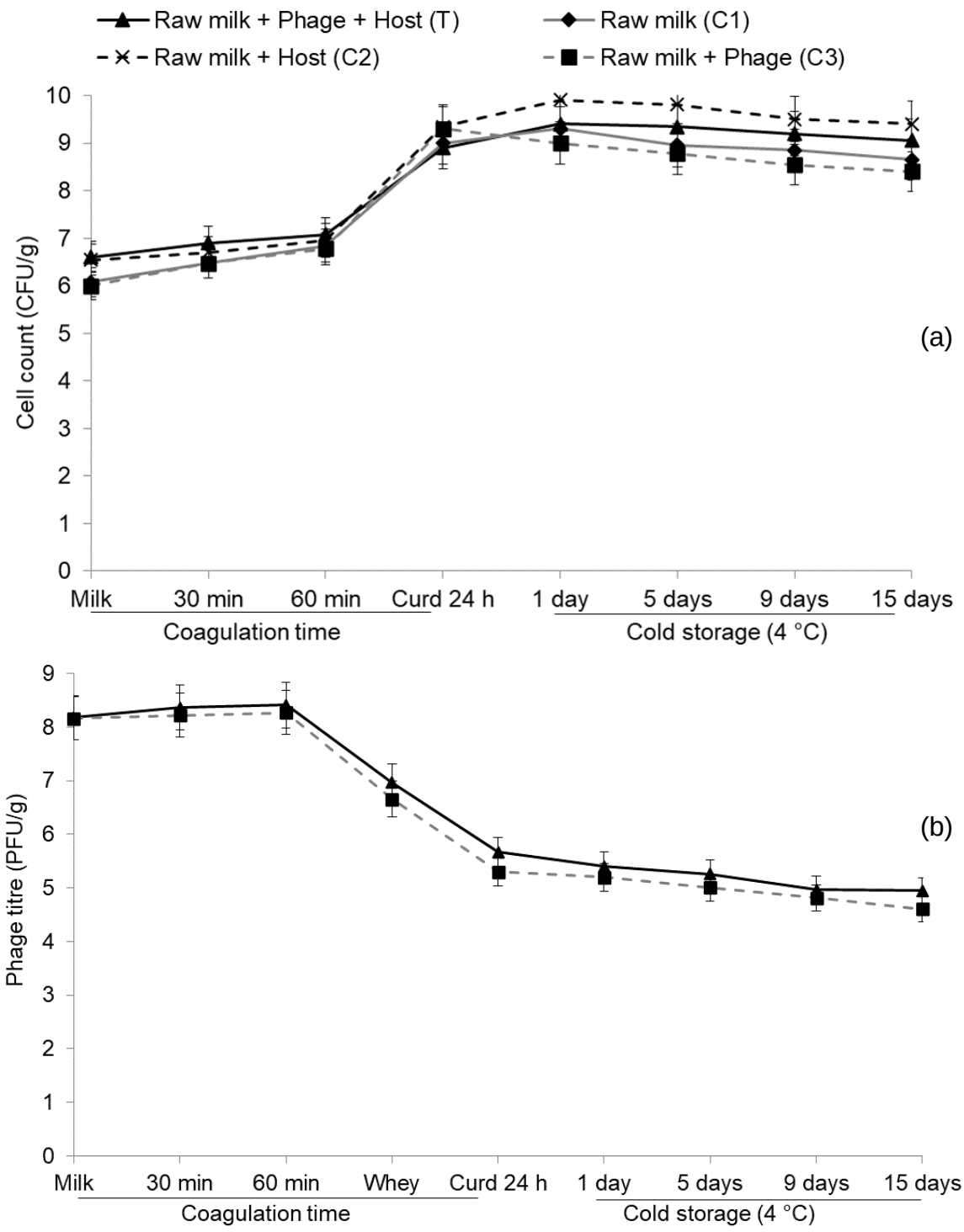

Figure 8.3. Total bacterial count (a) and *E. coli* phage T4 titre (b) screening throughout Karish cheese manufacture and cold storage. Data reported are means ± standard deviations of three independent trials with duplicate sampling and measurements.

8.3.1.2 *S. aureus* biocontrol during Karish manufacture and storage

The *S. aureus* phage cocktail efficacy to control the growth of inoculated (*S. aureus* DSM 104437 and ATCC 19685) and naturally present *S. aureus* and *Staphylococcus* spp. in Karish cheese is shown in Figure 8.4.

Although *S. aureus* in raw milk-plus host (C2) did not increase significantly (4.4 log CFU/g) at day 1 of storage, *S. aureus* showed high survival with small decrease ($p = 0.205$) until the end of 15 days (3.9 log CFU/g). This implies the ability of *S. aureus* strains to survive all the technological processes of Karish cheese. In curd (T; after 24 h), *S. aureus* phage cocktail was able to reduce *S. aureus* viable count to undetected level (≤ 10 CFU/g) and no re-growth was found after 1, 5, 9 and 15 days of cheese storage (Figure 8.4a). Similar results achieved in raw milk-plus-phage control (C3) demonstrate the phage cocktail's ability to eliminate inoculated and indigenous *S. aureus*. These data match previous results for phage cocktail treatment in raw skimmed milk after 24 h at 37 °C. Given the recorded broad host range (section 6.3.3), the phage cocktail was able to inhibit naturally occurring *Staphylococcus* spp. significantly ($p \leq 0.05$) to log 1.5 CFU/g in curd and complete elimination was occurred after 5 days till the end of storage (Figure 8.4b).

In addition, the total bacterial count screening during manufacture and storage (Figure 8.5a) showed a high significant ($p \leq 0.05$) increase from 6.3 (milk) to 8.8 (1 day) log CFU/g in treatment (T) and gradual decrease ($p = 0.863$) to 8.5 log CFU/g at the end of storage. Similar observations were recorded for the three controls and previously noted for *E. coli* phage application. Similar to what was previously observed with *E. coli*, the added starter culture had a noticeable effect on *S. aureus* growth when comparing raw milk-plus-host control (without starter culture) to C2. *S. aureus* viable count increased to 7.0 log CFU/ml in the absence of starter after 24 h of incubation at 37 °C. Accordingly, the *S. aureus* count reduction in untreated Karish, would be much related to the effect of starter and fermentation process.

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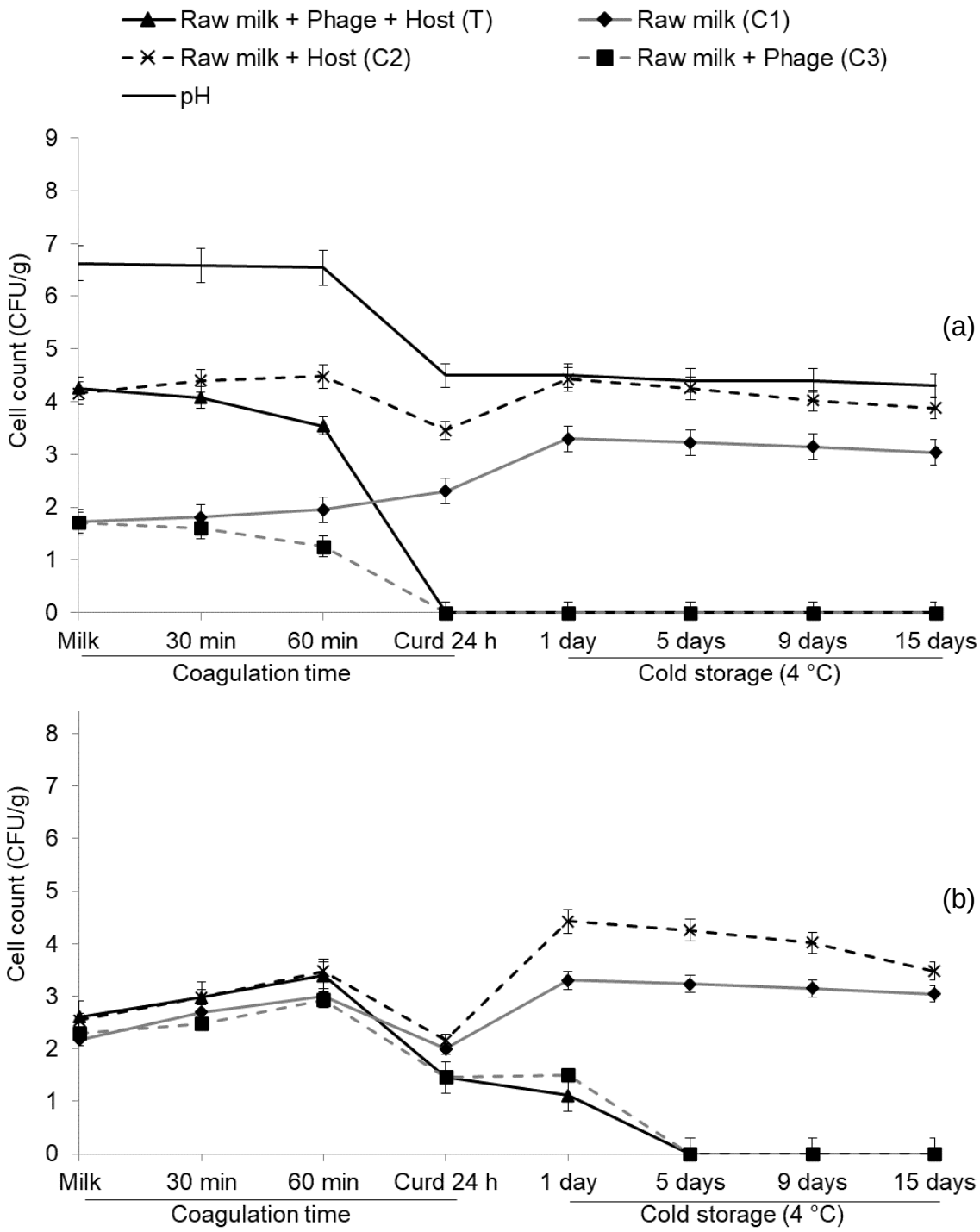

Figure 8.4. Effect of *S. aureus* phage cocktail (10^8 PFU/ml) on (a) inoculated and indigenous *S. aureus* and (b) indigenous *Staphylococcus* spp. growth during Karish cheese manufacture and cold storage. Data reported are means ± standard deviations of three independent trials with duplicate sampling and measurements.

Figure 8.5b demonstrates that the phage titres in treatments and raw milk-plus-phage controls decreased significantly ($p \leq 0.05$) to 3.5 ± 0.2 log PFU/g in curd, and no noticeable reduction occurred until the end of 15 days (3.5 ± 0.2 log PFU/g). This decline in phage titre in curd after 24 h could be related to the recorded loss of phage in whey (4.7 ± 0.2 log PFU/ml) and negative effect of low curd pH on phage survival. The stability of phage cocktail during cheese storage could be linked to their ability survive at low pH value (4.2), when previously tested in LB broth media.

The potential success of bacteriophages for controlling *S. aureus* in fresh acid cheese was previously studied by Bueno *et al.* (2012) who tested two *S. aureus* phage cocktails (vB_SauS-phi-IPLA35 and vB_SauS-phi-SauS-IPLA88; 10^6 PFU/ml) against *S. aureus* Sa9 (10^6 CFU/ml) in acid coagulated (pH ~4.2) fresh cheese made from pasteurized milk (with 10^7 CFU/ml mixed starter and 1.5 % dry cheese salting). The authors recorded a complete elimination of *S. aureus* after 6 h in presence of phage, however in phage absence the same result was recorded in curd after 24 h, which questioned the level of starter culture participation in reducing the *S. aureus* growth with phage treatment. Also, in concurrence with our results, Bueno *et al.* (2012) added that the phage cocktail was reduced by 3.0 and 4.0 log PFU/g in curd and after 14 days of storage (4 °C), respectively. In addition, no effect of phage treatment on cheese fermentation and physicochemical conditions was recorded.

Generally, lactic acid bacteria would reduce foodborne pathogen growth, although the rate of reduction is varied. Allam *et al.* (2017) demonstrate that using a *Lactococcus lactis* subsp *cremoris* (KM746) and *Lactococcus lactis* subsp *lactis* (KM721) (10^6 – 10^7 CFU/ml) starter was able to prevent coliform and *S. aureus* growth in Karish cheese made from pasteurized milk. Additionally, Viçosa *et al.* (2018) found that *Enterococcus faecalis* co-culture was able to hinder staphylococcal enterotoxin production in sterile skimmed milk (inoculated by 10^3 CFU/ml *S. aureus*) during 24 h of incubation at 30 °C. Accordingly, the achieved *S. aureus* elimination in curd before cheese storage was substantial to prohibit enterotoxins production in milk, that could otherwise withstand manufacture and subsequently be a serious problem in final cheese product (Mehli *et al.*, 2017).

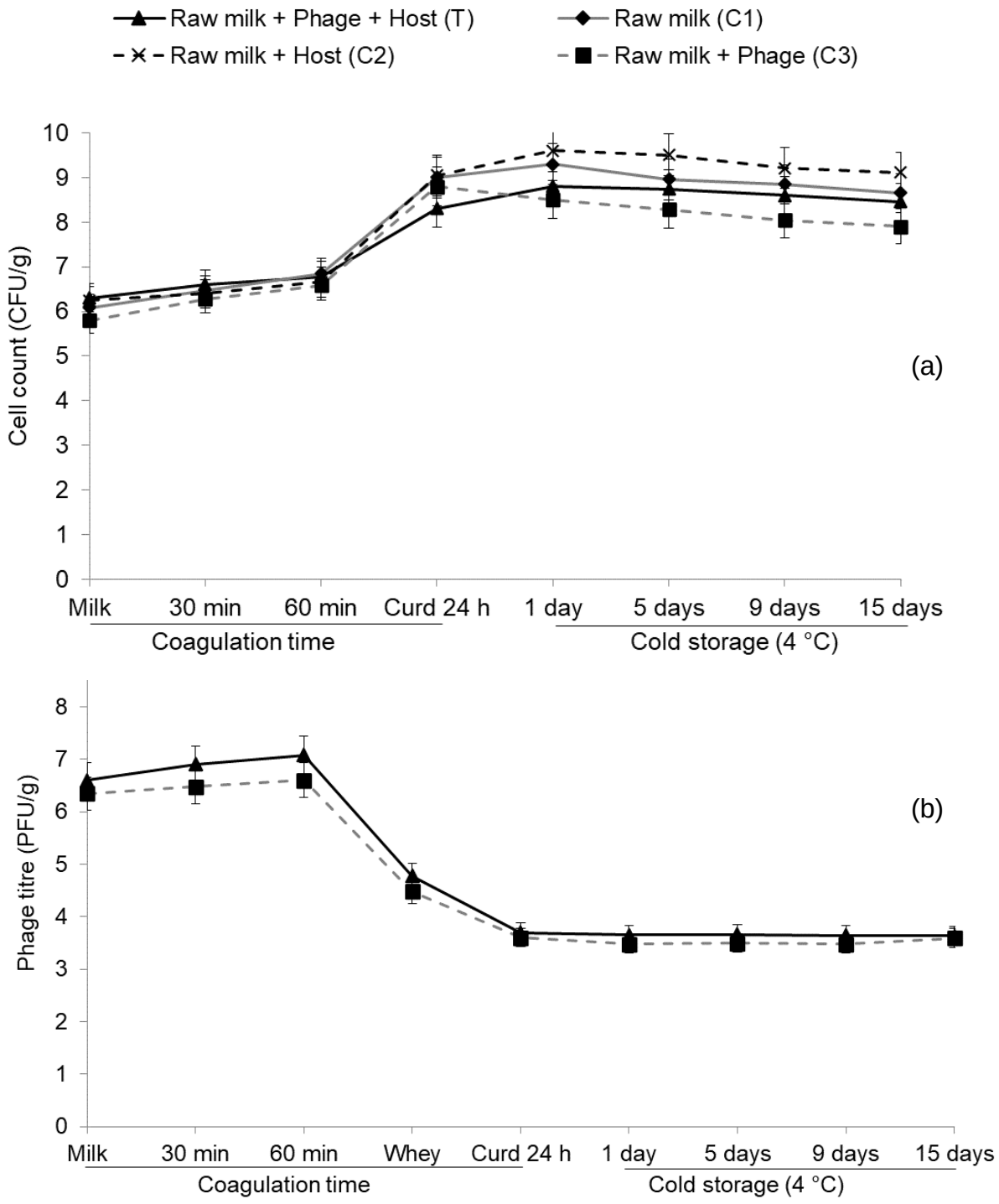

Figure 8.5. Total bacterial count (a) and *S. aureus* phage cocktail titre (b) screening throughout Karish cheese manufacture and cold storage. Data reported are means ± standard deviations of three independent trials with duplicate sampling and measurements.

8.3.2 Phage biocontrol activity in Domiati cheese

The efficacy of *E. coli* phage T4 and *S. aureus* phage cocktail (10^8 PFU/ml) to control host strains (10^4 CFU/ml) were independently tested during Domiati coagulation time and up to 30 days of ripening at 4 °C. The fermentation and enzymatic coagulation process was very similar in all treatments and matching the previously achieved results during Domiati cheese small scale manufacture (section 5.3.2).

8.3.2.1 *E. coli* biocontrol during Domiati manufacture and ripening

The *E. coli* phage T4 efficacy to control the growth of inoculated (*E. coli* NCIMB 10243) and naturally present *E. coli* in Domiati cheese, is shown in Figure 8.6. Although there was no recorded increase in *E. coli* viable count in both C1 and C2 controls, 2.0 and 2.8 log CFU/g *E. coli* was detected in C1 and C2, respectively, at the end of ripening. Interestingly, phage treatment (T and C3) reduced inoculated and indigenous *E. coli* below the detection limit (≤ 10 CFU/g) from 1 to 30 days of ripening in brine solution at 4 °C.

As the pH did not significantly decrease from 6.7 (milk) to 6.5 (curd) until the end of 30 days (pH= 6.3) of ripening, it is clear that the fermentation process had no noticeable influence on *E. coli* growth or phage activity. The inability of *E. coli* to grow in milk during coagulation time could be attributed to the high salt concentration in milk at the beginning of manufacture. Salting in Domiati cheese was done directly in milk gave around 8.0 % in milk and decreased to 4.2 % in curd after whey separation, increasing gradually again to 6.0 % after 42 days of ripening in brine solution (section 5.3.2). Similarly, same observation could be noted when comparing raw whole milk-plus-host control (without salting and starter culture) to C2. As *E. coli* viable count increased to 8.3 log CFU/ml in absence of salting and added starter after 24 h of incubation at 37 °C. Accordingly, the decline in *E. coli* count during Domiati cheese ripening time would be much related to the antagonistic effect between phage and salt concentration.

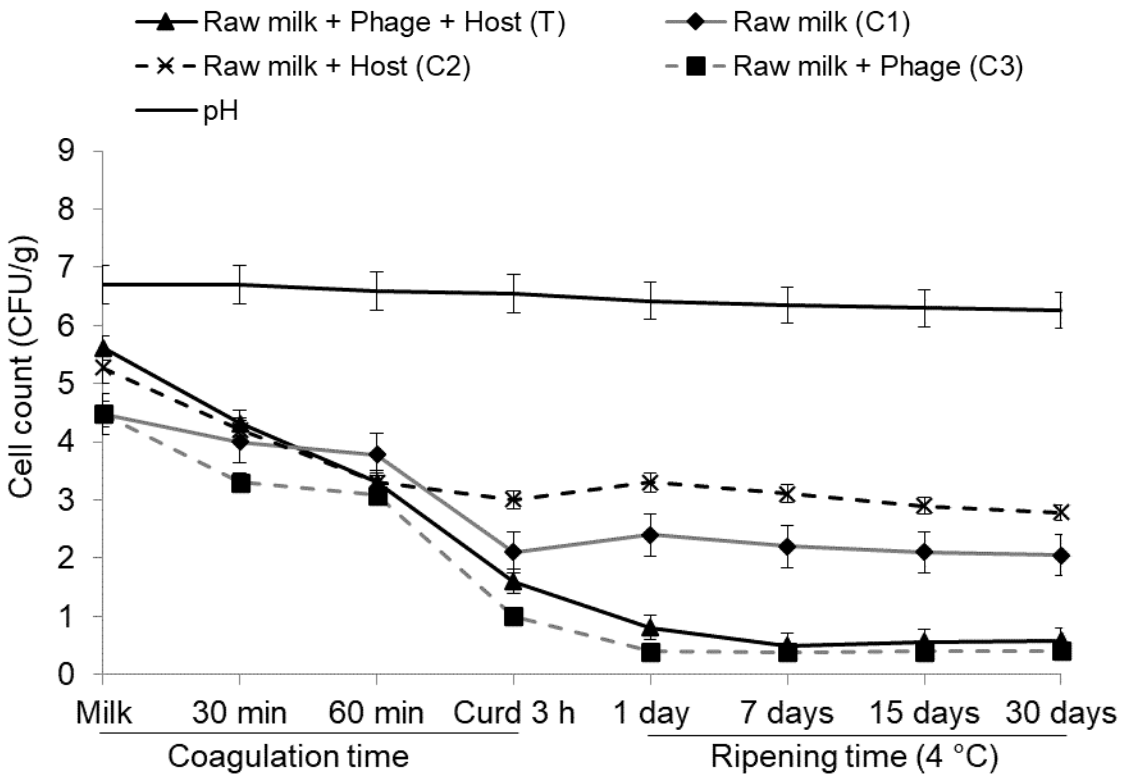

Figure 8.6. Effect of *E. coli* phage T4 (10^8 PFU/ml) on inoculated and indigenous *E. coli* growth throughout Domiati cheese manufacture and ripening. Data reported are means ± standard deviations of three independent trials with duplicate sampling and measurements.

In addition, the total bacterial count screening during manufacture and storage (Figure 8.7a), showed no significant (p = 0.294) increase from 6.6 (milk) to 7.1 (curd) log CFU/g in treatment (T) and increased ($p \leq 0.05$) to 7.6 log CFU/g at day 1, then decreased gradually to 5.5 log CFU/g at the end of ripening time. Similar observations were recorded for the three controls. The increase of TBC at day 1 could be regarded to the concentration of strains after complete whey separation.

Moreover, the phage titres in treatment and raw milk-plus-phage control were decreased significantly ($p \leq 0.05$) to 3.7 ± 0.2 log PFU/g in curd, and further reduction (p = 0.691) occurred until the end of 30 days (3.2 ± 0.2 log PFU/g) (Figure 8.7b). As noted with Karish cheese, the decline in phage titre in curd after 3 h could be attributed to the recorded loss of phage in whey (4.0 ± 0.1 log PFU/g). Conversely, the stability of phage titre during the cheese ripening at average salt of 5.0 % and pH of 6.4, implies their ability to keep the *E. coli* count below detection limit after ripening time and during cheese storage.

Of note, *E. coli* phage T4 showed a high ability to infect the target strain within used high salt concentration. These results are in accordance with Fister *et al.* (2016b) who stated that *L. monocytogenes* phage (P100) and host bacteria interaction was not affected, when tested in TSB media with 2 M NaCl during 24 h of incubation at 37 °C. However, P100 phage replication was more affected by NaCl concentration. Fister *et al.* (2016b) added that phage replication was more dependent to optimum growth conditions for the host bacteria. Within this approach, Guenther *et al.* (2009) revealed that *L. monocytogenes* phage (A511; 10^8 PFU/ml) completely eliminated *L. monocytogenes* strains (WSLC 1001; 10^3 CFU/ml) after 3 days of storage in mozzarella cheese brine at 6 °C.

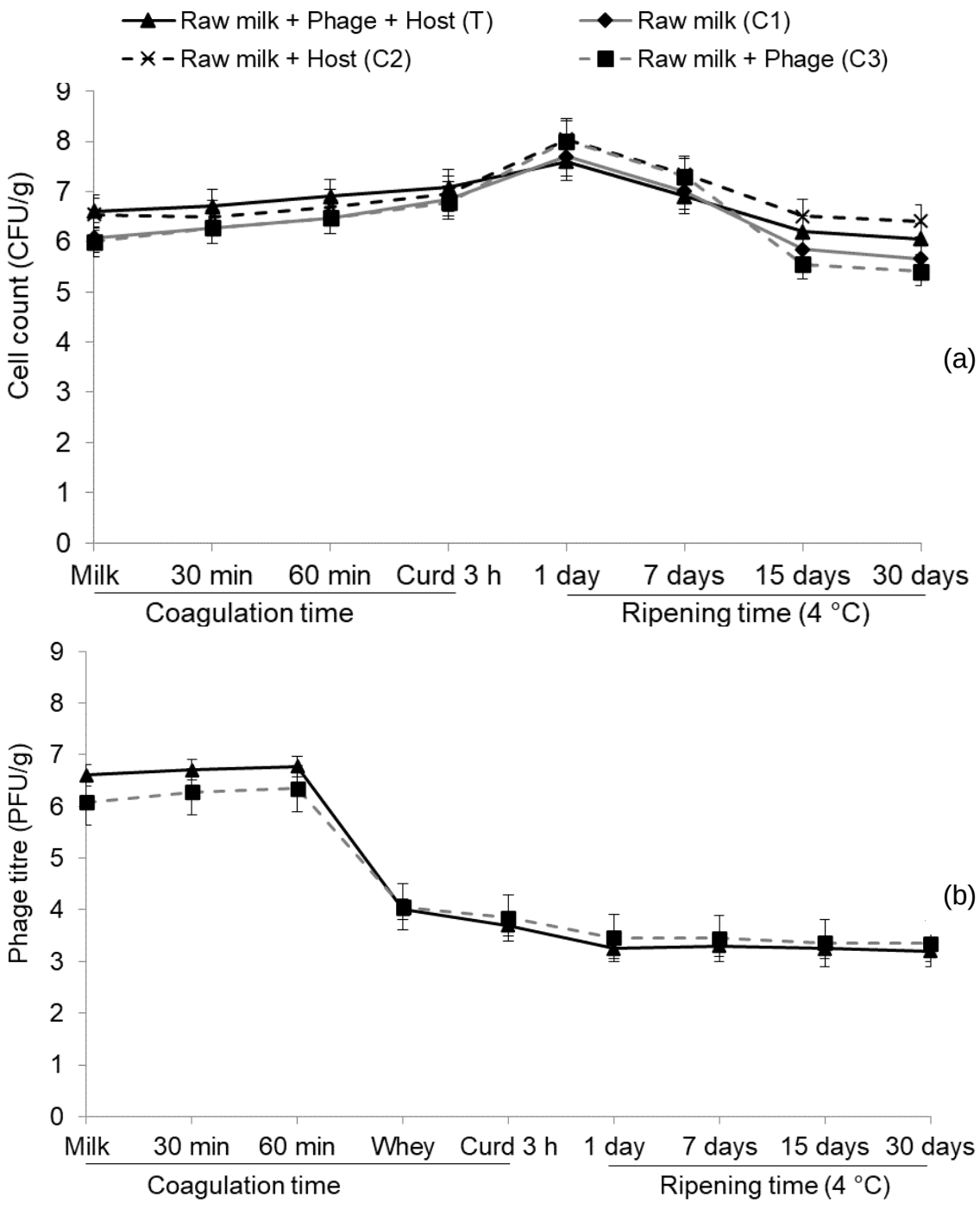

Figure 8.7. Total bacterial count and *E. coli* phage T4 titre screening throughout Domiati cheese manufacture and ripening. Data reported are means ± standard deviations of three independent trials with duplicate sampling and measurements.

8.3.2.2 *S. aureus* biocontrol during Domiati manufacture and ripening

The *S. aureus* phage cocktail efficacy to control the growth of inoculated (*S. aureus* DSM 104437 and ATCC 19685) and naturally present *S. aureus* and *Staphylococcus* spp. in Domiati cheese is shown in Figure 8.8.

Although there was no significant increase (p= 0.699) in *S. aureus* viable count during coagulation in both C1 and C2 controls, the count increased ($p \leq 0.05$) at day 1 to 3.3 and 5.3 log CFU/g *S. aureus* in C1 and C2, respectively, at the end of ripening. In curd (T; after 3 h), *S. aureus* phage cocktail was able to reduce *S. aureus* viable count to undetected level (≤ 10 CFU/g) and no re-growth was found after 1, 7, 15 and 30 days of cheese ripening in brine solution (Figure 8.8a). Likewise, phage cocktail showed same activity in raw milk-plus-phage control (C3), that demonstrate the phage cocktail ability to target both inoculated and indigenous *S. aureus*. These data are matching the previous results in Karish cheese (Figure 8.4) and raw whole milk after 24 h at 37 °C. Due to the broad host range (section 6.3.3), the phage cocktail was able to significantly reduce ($p \leq 0.05$) naturally occurring *Staphylococcus* spp. to log 1.3 CFU/g in curd and completely eliminated after 7 days till the end of storage (Figure 8.8b).

Similar to what was observed with *E. coli* treatment, the fermentation process showed no noticeable influence on *S. aureus* growth or phage activity. The ability of *S. aureus* to grow at high salt concentration (8.0 %) exerted during milk coagulation time, was previously observed in *S. aureus* phage EBHT testing in LB broth with 4.0 and 8.0 % salt after 24 h at 37 °C. However, the *S. aureus* growth rate was negatively affected by high salt concentration, as noted when comparing raw whole milk-plus-host control (without salting and starter culture) to C2. *S. aureus* viable count increased to 5.8 log CFU/ml in absence of salting and added starter after 2 h of incubation at 37 °C.

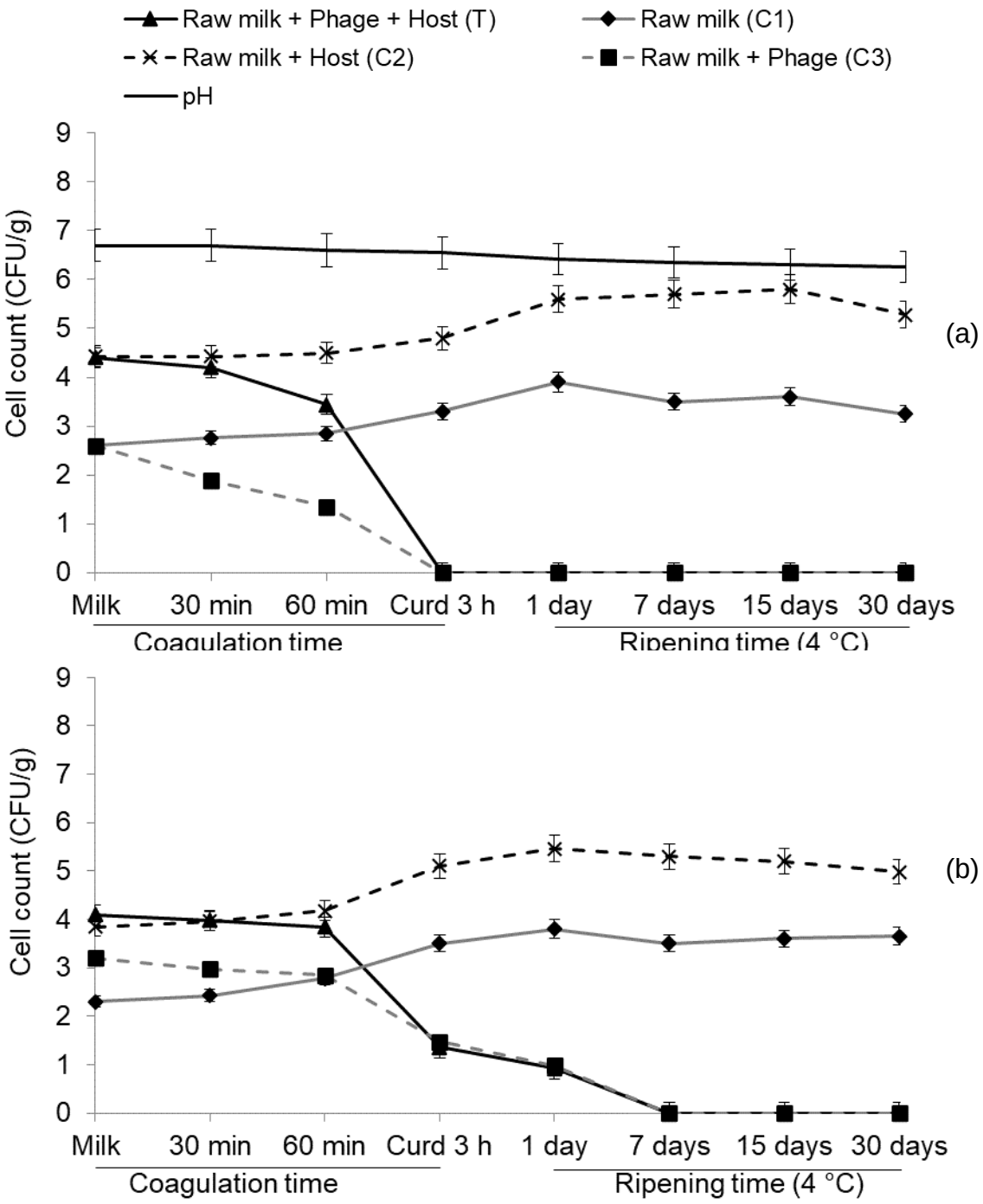

Figure 8.8. Effect of *S. aureus* phage cocktail (10^8 PFU/ml) on inoculated and indigenous *S. aureus* (a) and indigenous *Staphylococcus* spp. (b) in Domiati cheese manufacture and ripening. Data reported are means ± standard deviations of three independent trials with duplicate sampling and measurements.

However, *S. aureus* phage cocktail completely eliminated *S. aureus* after 24 h in raw whole milk (without salt or starter), the same effect was recorded after 4 h of inoculation in Domiati curd. That showed that the decline in *S. aureus* count during Domiati cheese coagulation time would be much related to the synergistic effect between phage and salt concentration. However, it would be recommended to increase the testing intervals (between 2 and 24 h) in raw whole milk to understand the exact point of complete pathogen inhibition.

These results can be linked to García *et al.* (2007), who investigated the biocontrol activity of two *S. aureus* phages (Φ88 and Φ35) in combination at 10^8 PFU/ml to target *S. aureus* Sa9 (10^6 CFU/ml) in rennet curd processing from pasteurized whole milk. The authors found that the phage cocktail elimianted *S. aureus* in milk after 1 h of incubation at 30 °C prior to addition of rennet enzyme. However, the obtained curd was not processed further to cheese and stored at 4 °C.

On the other hand, the total bacterial count screening during manufacture and storage (Figure 8.9a) showed slow log CFU/g decrease from 7.8 (milk) to 7.0 (curd) in treatment (T) and decreased ($p = 0.863$) gradually to 5.3 log CFU/g at the end of ripening; similar observations were recorded for the three controls. These results demonstrate the lower ability of added starter to grow at the high salt level, which was reflected in the pH development during coagulation time and ripening. These obtained data are in concurrence with Al-Nabulsi *et al.* (2020) who stated that inoculated starter (5.0 log CFU/ml; *Streptococcus thermophilus* and *Lactobacillus bulgaricus*) was not able to grow and decreased during production of white-brined cheese (10.0 % salt) up to 28 days of storage at 10 °C. However, this observation is in contrast to Ayad (2009) findings, where 1.5 ± 0.4 log CFU/g increase in starter (*Lactococcus lactis* and *Lactococcus cremoris*) count were recorded during Domiati cheese (5.0 % salt) manufacture until 3 months at 10 °C. Therefore, the starter culture development and fermentation in Domiati would be more linked to starter species, inoculation level and salt concentration.

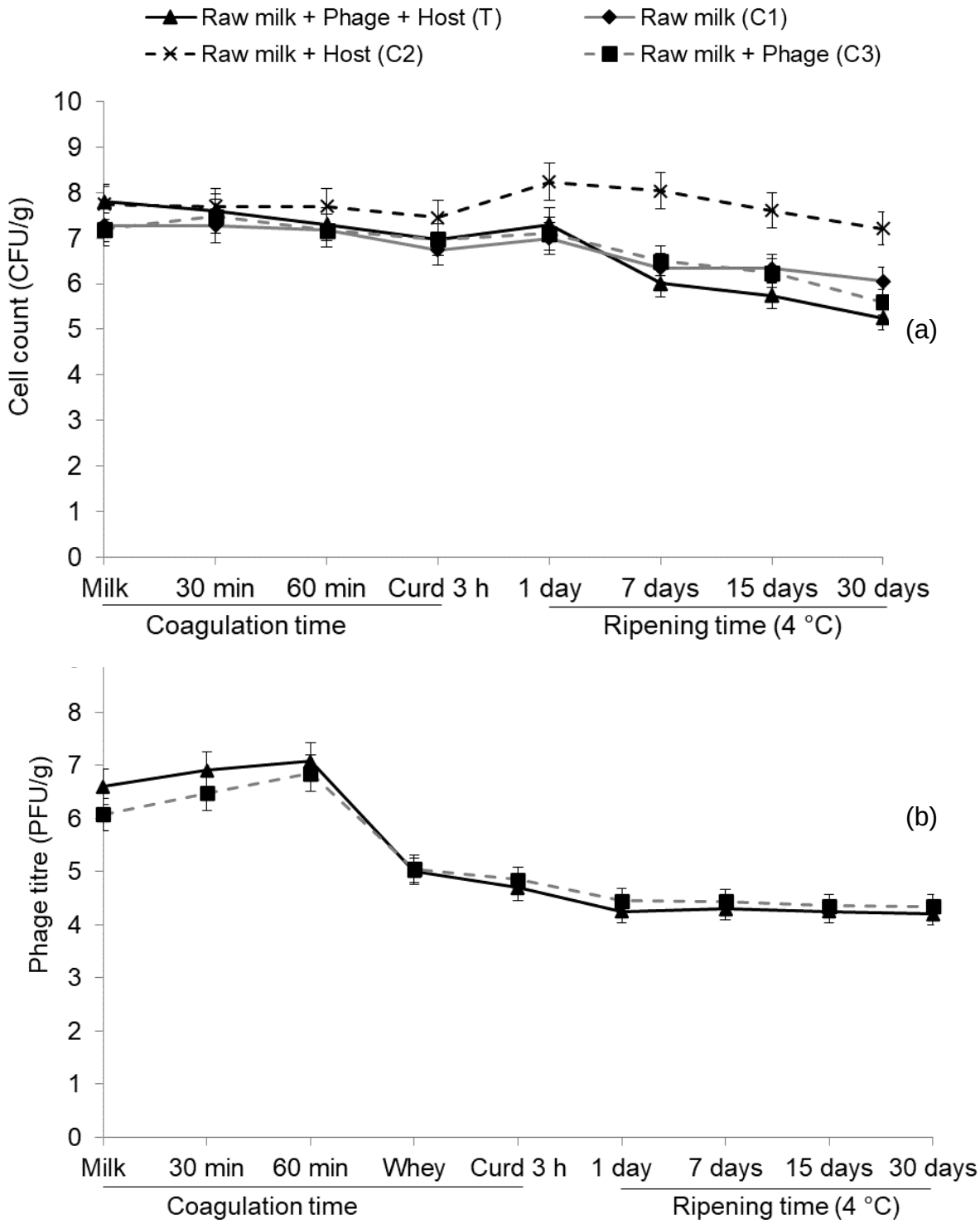

Figure 8.9. Total bacterial count (a) and *S. aureus* phage cocktail titre (b) screening throughout Domiati cheese manufacture and ripening. Data reported are means ± standard deviations of three independent trials with duplicate sampling and measurements.

Figure 8.9b showed that the phage titres in treatment and raw milk-plus-phage control were decreased significantly ($p \leq 0.05$) to 4.7 ± 0.1 log PFU/g in curd, and no significant reduction occurred until the end of 30 days (4.2 ± 0.1 log PFU/g). The decline in phage titre in curd after 24 h as previously observed could be related to phage loss in whey (5.0 ± 0.1 log PFU/ml). The stability of phage cocktail during cheese ripening could be linked to the recorded phage ability to survive at 4.0 and 8.0 % salt concentrations in LB broth media,

As Domiati cheese can be consumed fresh or mainly after 30 days of ripening in brine solution, the presence of 5.2 log CFU/g *S. aureus* at the end of cheese (C2) ripening raises a serious concern of enterotoxins production, assuming that presence of 10^3 to 10^5 CFU/ml of *S. aureus* in milk would be sufficient for enterotoxin production (Commission Regulation [EC] No., 2073/2005). Accordingly, the achieved *S. aureus* elimination in curd before cheese storage was substantial to prohibit *S. aureus* growth and the probability of enterotoxins production in milk.

8.3.3 Phage biocontrol activity in Ras cheese

The efficacy of *E. coli* phage T4 and *S. aureus* phage cocktail (10^8 PFU/ml) to control host strain (10^4 CFU/ml) was independently tested during Ras coagulation time and up to 90 days of ripening at 9 - 12 °C and 85 % RH. The fermentation and enzymatic coagulation process was very similar in all treatments and matching the previously achieved results during Ras cheese small scale manufacture (section 5.3.3).

8.3.3.1 *E. coli* biocontrol during Ras manufacture and ripening

The *E. coli* phage T4 efficacy to control the growth of inoculated (*E. coli* NCIMB 10243) and naturally present *E. coli* in Ras cheese, is shown in Figure 8.10. Inoculated and indigenous *E. coli* population increased in C2 (raw milk plus-host) from 5.3 log CFU/g in milk to 6.6 log CFU/g in curd, whereas, *E. coli* population decreased in cheese gradually to 4.3 log CFU/g after 90 days. Phage treatment (T) reduced ($p \leq 0.05$) inoculated and indigenous *E. coli* from 5.5 (milk) to 2.8 log CFU/g in curd (after 45 min). The *E. coli* count was slightly increased ($p = 0.153$) at day 1 of storage (2.9 log CFU/g), and a continuous reduction ($p \leq 0.05$) to 2.0 log CFU/g was achieved at the end of 90 days of storage. Likewise, the obtained *E. coli* reduction in raw milk-plus-phage control (C3), confirming the ability of the phage to target the naturally present *E. coli*. However, it was noted that the phage biocontrol activity was lower compared to what previously achieved with Karish (section 8.3.1.1) and Domiati (section 8.3.2.1).

E. coli strains showed high ability to grow and survive during fermentation process which reduced pH from 6.6 (milk) to 6.3 (curd) and 5.9 (day 1) until the end of 90 days (pH= 4.9) of ripening. This is in accordance with previous results (section 6.3.2), that showed a high ability of *E. coli* T4 host to grow with high phage lytic ability at 5.9 and 5.1 pH in LB broth media during 24 h of incubation at 37 °C. In addition, the presence of 3.9 ± 0.1 % salt (section 5.3.3) in cheese reduced *E. coli* growth rate without affecting phage lytic ability when previously tested in broth media.

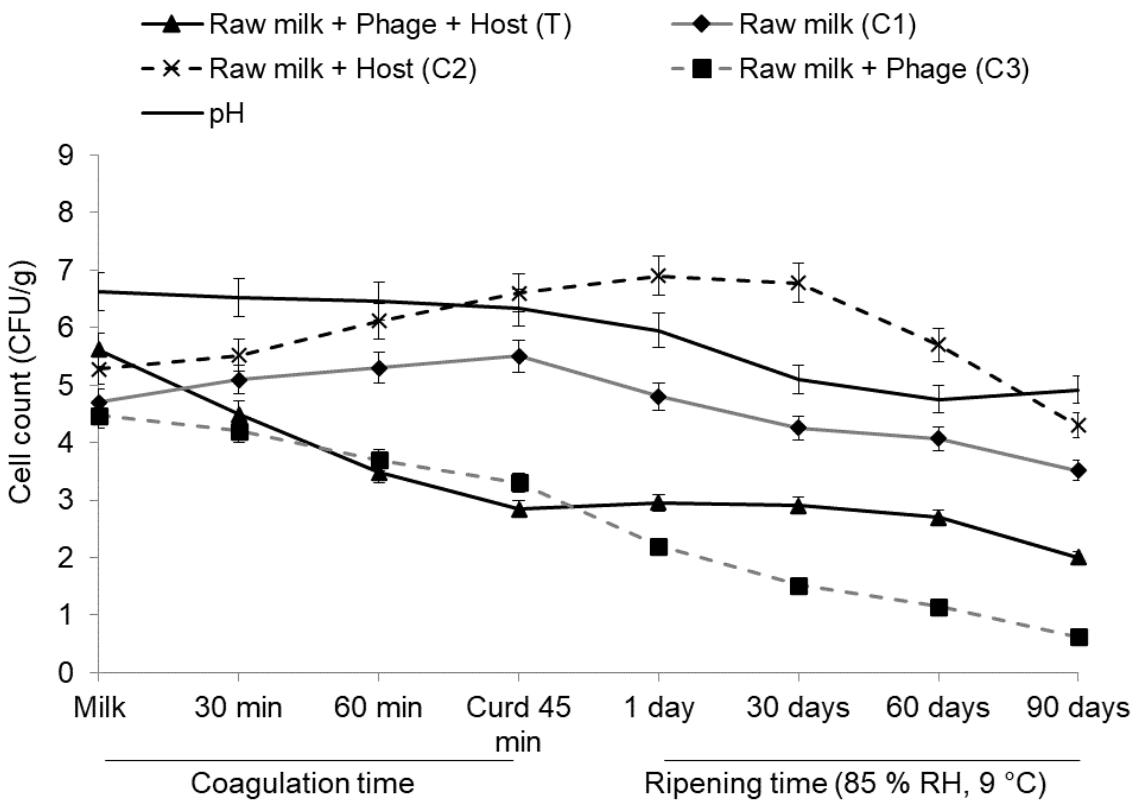

Figure 8.10. Effect of *E. coli* phage T4 (10^8 PFU/ml) on inoculated and indigenous *E. coli* growth throughout Ras cheese manufacture and ripening. Data reported are means ± standard deviations of three independent trials with duplicate sampling and measurements.

It is noteworthy that the added starter culture did not show a noticeable effect on *E. coli* growth in all controls and treatment (T), when comparing phage T4 results in raw whole milk after 2 h (6.2 log CFU/ml; without starter culture) to these data. However, the decline in *E. coli* viable count (C2) was only recorded after 30 days of ripening, whereas it was noted at day 1 in C1. These observations could be related to the reduction of moisture content with increasing salt content (salting was done at the first 15 days of ripening) that led to reduce water activity.

In addition, the total bacterial count screening during manufacture and storage (Figure 8.11a) showed a non-significant increase log CFU/g from 6.5 (milk) to 6.9 (curd) in treatment (T) and a large increase ($p = 0.001$) to 8.9 log CFU/g at day 1. However, TBC then decreased ($p \leq 0.05$) gradually to 6.4 log CFU/g after 90 days of ripening; similar observations were recorded for the three controls. The remarkable TBC increase at day 1 could be related to starter growth during scalding (40 – 50 min), embedded bacterial cells in enzymatic curd matrix and concentration factor due to whey separation (Bueno *et al.*, 2012). Additionally, the decline in TBC may be related to LAB cell autolysis as part of ripening process (Khattab *et al.*, 2019), however further research would be required to examine the effect of phage treatment on hard cheese ripening process.

Figure 8.11b, demonstrated that phage titres in treatment and raw milk-plus-phage control were highly decreased ($p \leq 0.05$) to 4.3 ± 0.2 log PFU/g in curd, and 3.2 ± 0.1 log PFU/g at day 1. Although phage titre showed good stability (3.2 ± 0.1 log PFU/g) after 30 days, further reduction ($p = 0.009$) occurred until the end of 90 days (2 ± 0.2 log PFU/g).

Declining phage T4 biocontrol activity could be attributed to many factors as follows: a) the time needed for phage to reveal highest lytic ability was not enough. According to phage testing in raw whole milk, a minimum 2 h was required to chieve highest lytic ability, where 1 h was available for phage T4 to work in liquid milk; b) increasing temperature from 32 to 45 °C during curd scalding could have caused a shock to phage and host, which could make host strain more resistant to antimicrobial treatment (Tabla *et al.*, 2022).

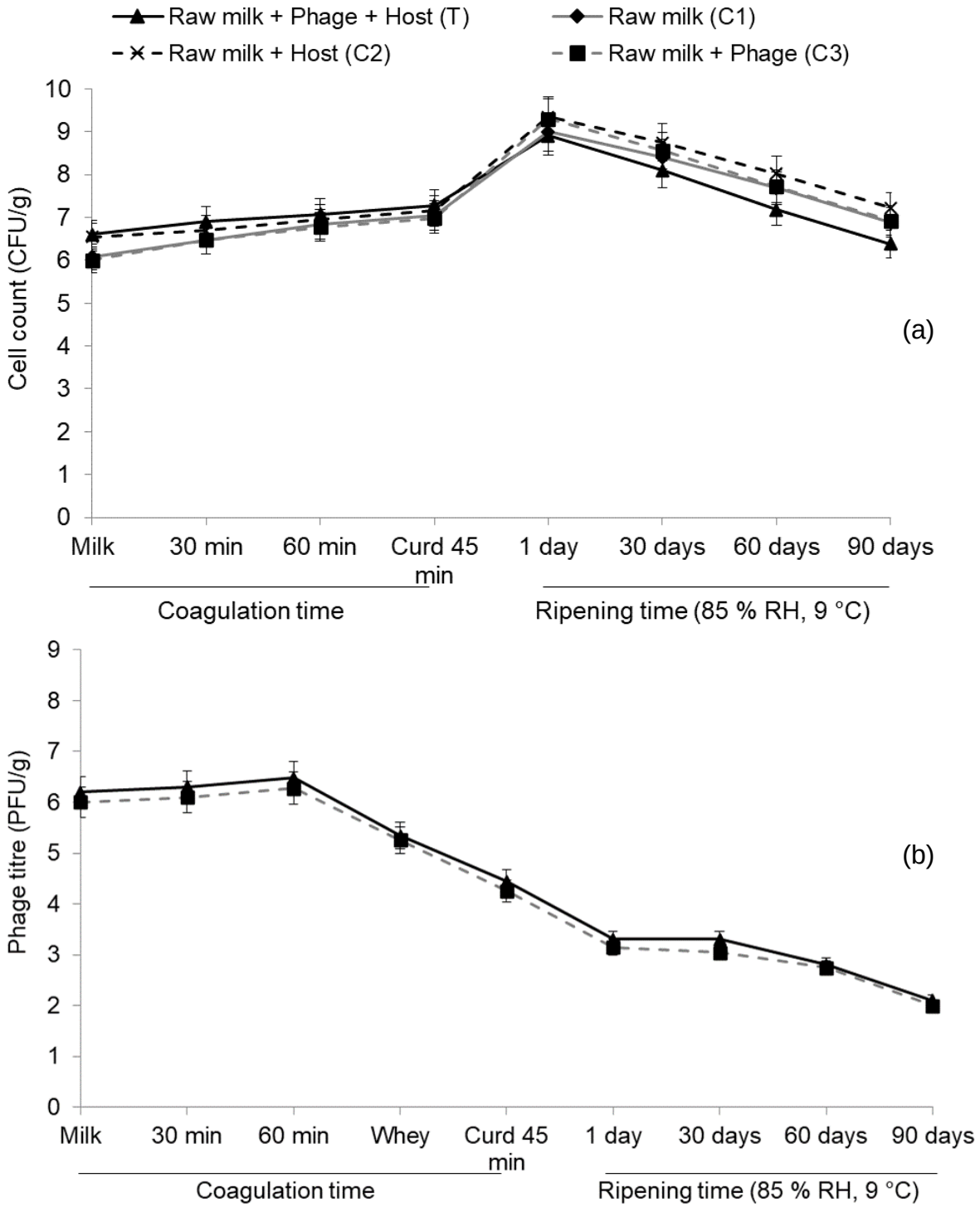

Figure 8.11. Total bacterial count (a) and *E. coli* phage T4 titre (b) screening throughout Ras cheese manufacture and ripening. Data reported are means ± standard deviations of three independent trials with duplicate sampling and measurements.

High phage titre (c) loss during whey separation; as Ras is a hard cheese, low moisture content (35 ± 4 %) was detected during ripening (section 5.3.3). Conversely, the low stability of phage titre during Ras manufacture and ripening, questions their ability to keep the *E. coli* count at low level or fight any outer contamination. Accordingly, the decline in *E. coli* count during Ras cheese ripening, would be much related to the antagonistic effect between starter, phage, moisture and salt.

A recent study by Tabla *et al*. (2022) studied the influence of *E. coli* phage cocktail (10^7 PFU/ml; Φ565.2, Φ565.1, Φ13 and Φ3) on *E. coli* RT1 to reduce early blowing development in semi-hard cheese made from pasteurized whole goat milk and starter culture mix (10^6 CFU/ml). Of note, the manufacture process of previously mentioned cheese is close to Ras cheese. The authors found that phage treatment decreased *E. coli* count gradually to aproximately log 1.0 log CFU/g after 60 days of ripening. However, phage treatment was not able to decrease *E. coli* development in curd, and complete *E. coli* inhibition was also recorded in control (without phage) after 60 days. The authors added that the cheese technological process may hindered phage activity and more time between phage inoculation and curdling is required. In accordane to our results, Tabla *et al.* stated that there was a recorded effect of starter on *E. coli* growth.

In a different prespective, Modi *et al.* (2001) compared the efficacy of *Salmonella* Enteritidis SJ2 phage (10^8 PFU/ml) against host (10^4 CFU/ml) in chedder cheese made from raw and pasteurized milk. The authors found that phage treatment reduced *Salmonella* Enteritidis by 3.0 and 4.0 log CFU/g in curd of pasteurized and raw milk, respectively, where around 10^3 CFU/g was still detected in cheese at first day. Moreover, a large reduction of target strain started after 39 days of ripening, that leading to complete *Salmonella* Enteritidis elimination after 89 days of pasteurized cheese ripening. However, this behaviour was not observed in raw milk cheese until 99 days. Notably, similar observations were found in C3 (Raw milk + Phage) and T (Raw milk + Phage + Host) (Figure 8.10), however a clear explaination of this behaviour is unknow. One reason that may clarify this behaviour is that phage adsorped to host in milk in the liquid state that cause later lysis in cheese.

8.3.3.2 *S. aureus* biocontrol during Ras manufacture and ripening

The *S. aureus* phage cocktail efficacy to control the growth of inoculated (*S. aureus* DSM 104437 and ATCC 19685) and naturally present *S. aureus* and *Staphylococcus* spp. in Ras cheese, is shown in Figure 8.12. *S. aureus* in raw milk-plus host (C2) significantly increased ($p \leq 0.05$) (5.7 log CFU/g) at day 1 of storage, and showed high stability with small decrease (p = 0.311; 5.3 log CFU/g) until the end of 90 days. This implies the ability of *S. aureus* strains to survive all the technological processes of Ras cheese. *S. aureus* phage cocktail was able to reduce *S. aureus* viable count from 4.4 log CFU/g (T; milk) to 2.5 log CFU/g in curd, where the *S. aureus* reduction continued to reach undetectable levels (≤ 10 CFU/g) after 60 and 90 days of ripening (Figure 8.12a). Similar results were achieved in raw milk-plus-phage control (C3), demonstrating the phage cocktail's ability to eliminate inoculated and indigenous *S. aureus*. Given the broad host range (section 6.3.3), the phage cocktail was able to reduce naturally occurring *Staphylococcus* spp. gradually from 4.1 log CFU/g (milk) to below detection limit (≤ 10 CFU/g) after 90 days compared to control (C2; 5.0 log CFU/g) (Figure 8.12b).

Similarly, the added starter culture did not have a noticeable effect on *S. aureus* growth in all controls and treatment (T), when comparing these data to phage cocktail results in raw whole milk after 1.5 h (5.5 log CFU/ml) without starter culture. However, the decline in *S. aureus* viable count (C1) was only recorded after 30 until 90 days. As mentioned in section 8.3.3.1, this observation could be related to reduced moisture content with increasing salt content. However, the susceptibility of *S. aureus* to these conditions was more strain dependent.

In addition, the total bacterial count screening during manufacture and storage (Figure 8.13a), showed non-significant log CFU/g increase from 7.1 (milk) to 7.6 (curd) in T and high increase ($p \leq 0.05$; 8.4 log CFU/g) was recorded at day 1. However, TBC then decreased ($p \leq 0.05$) gradually to 5.8 log CFU/g after 90 days of ripening. Similar observations were previously recorded and explained with phage T4 trials in section 8.3.3.1.

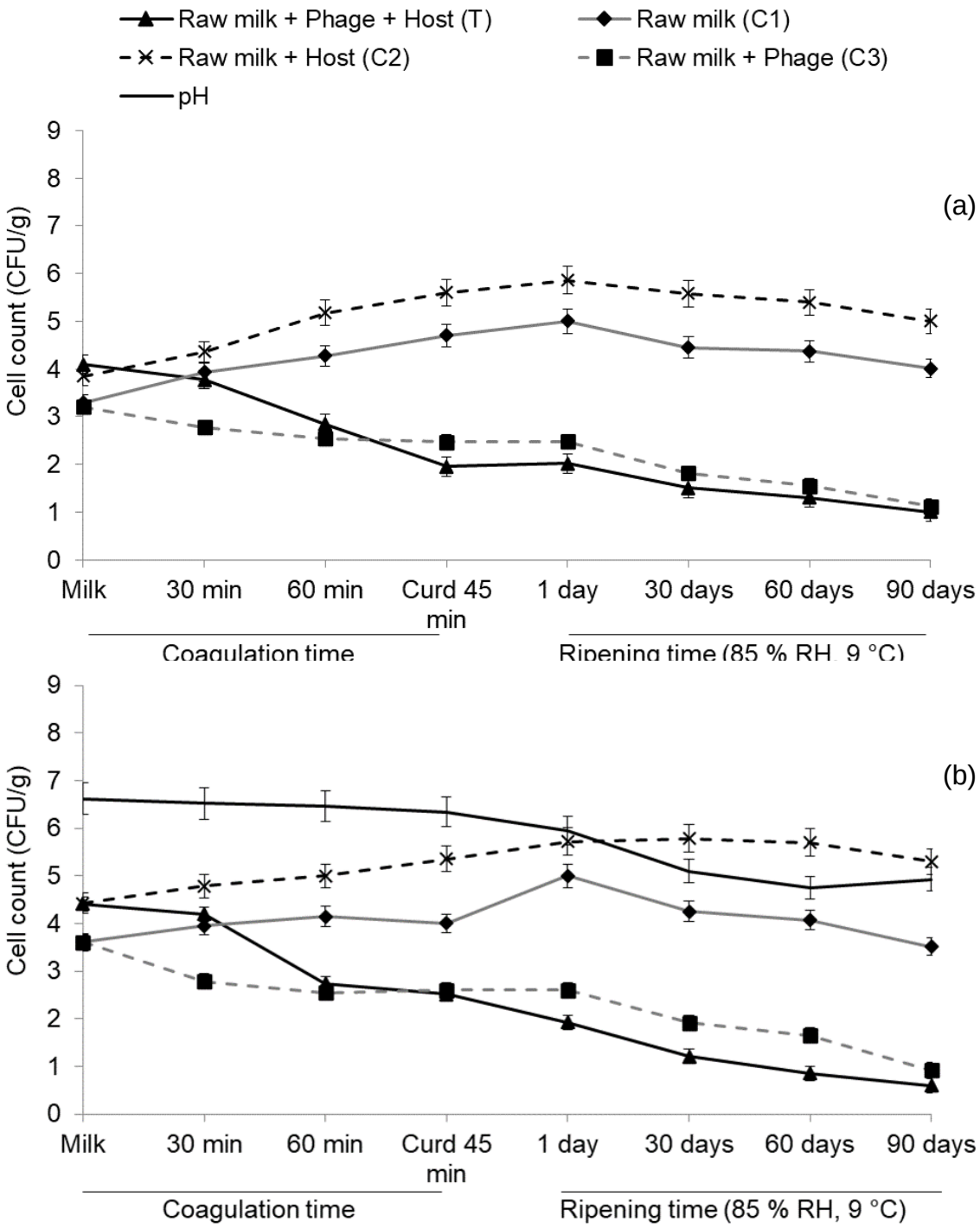

Figure 8.12. Effect of *S. aureus* phage cocktail (10^8 PFU/ml) on inoculated and indigenous *S. aureus* (a) and indigenous *Staphylococcus* spp. (b) growth throughout Ras cheese manufacture and ripening. Data reported are means ± standard deviations of three independent trials with duplicate sampling and measurements.

Figure 8.13b, demonstrated that the phage titres in treatment and raw milk-plus-phage control were significantly reduced ($p \leq 0.05$) to 4.5 ± 0.2 log PFU/g in curd, and 3.5 ± 0.3 log PFU/g at day 1. A continuous phage titre reduction occurred to 2.3 ± 0.1 log PFU/g after 90 days.

A number of studies have investigated the use of phages to inhibit *S. aureus* pathogen growth in semi-hard or hard cheeses. Bueno *et al*. (2012) found that inoculation of 10^6 PFU/ml phage cocktail against *S. aureus* Sa9 (10^6 CFU/ml) at the beginning of hard cheese manufacture led to a 4.7 log CFU/g reduction compared to control and 1.24 log CFU/g *S. aureus* was still detected. However, the same phage cocktail completely inhibited *S. aureus* growth after 6 h in acid soft cheese application. Further, according to El-Haddad *et al.* (2016) the application of a cocktail of two *S. aureus* phages (phi812, 44AHJD and phi2; 10^8 PFU/ml) reduced an initial 10^6 CFU/ml *S. aureus* viable count by 2.0 log CFU/ml in curd during cheddar-like production. Although lower phage concentrations (1×10^7 and 5×10^7 PFU/ml) reduced *S. aureus* by only 1 ± 0.5 log CFC/g in curd, a reduction to 2 log CFU/g were observed after 14 days of ripening at all phage concentrations.

Thus, in accordance with achieved results, reduced phage biocontrol activity in hard cheese production could be linked to the minimum ability of phages to diffuse or move within strong enzymatic casein micelles, which would subsequently hinder the ability of phage to face target strain and infection process (Modi *et al.*, 2001; Guenther and Loessner, 2011; LeLièvre *et al.*, 2019; Tabla *et al.*, 2022). However, the impact of food matrix on phage activity is varied according to the exact structure, composition and incorporated environmental conditions (Ly-Chatain, 2014).

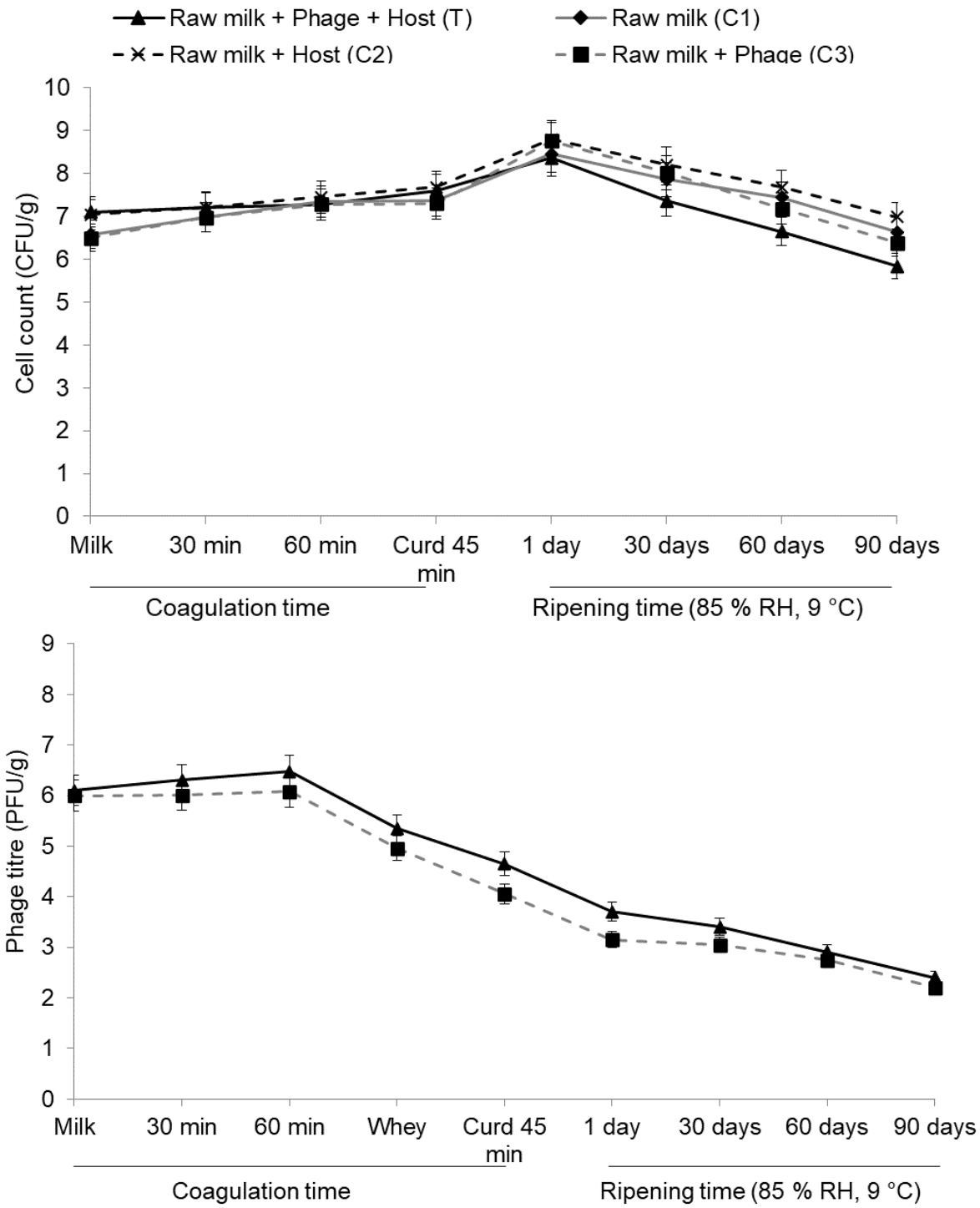

Figure 8.13. Total bacterial count (a) and *S. aureus* phage cocktail titre (b) throughout Ras cheese manufacture and ripening. Data reported are means ± standard deviations of three independent trials with duplicate sampling and readings.

The decline in *S. aureus* phage cocktail biocontrol activity could be attributed to the same mentioned factors for phage T4. Although phage cocktail was able to completely eliminate *S. aureus* in curd without recording any regrowth during Karish storage and Domiati ripening, it was not able to achieve the same activity in Ras. The unfavourable effect of Ras manufacture process on the phage cocktail used highlights the criticality of achieving pathogen inhibition in milk before entering the coagulation step. These data show that even if the phage applied has a high lytic ability, it would be difficult to tackle target strain in solid matrix (cheese).

Taken altogether, our results suggest that the high success of phage cocktail to decrease *S. aureus* to an undetectable level at the stage that Ras cheese is ready for consumption (after 90 days), would lower the risk of enterotoxin accumulation during cheese manufacture, ripening and subsequent storage. This assumption is supported by El-Haddad *et al.* (2016) who observed a reduction of staphylococcal enterotoxins production in cheese with phage treatment.

8.3.4 Influence of cheese technological process on phage titre

Karish, Domiati and Ras cheeses have varied physicochemical properties, linked to different strict technological processes. Changes in one critical manufacture step could lead to changes in final cheese product characteristics. Therefore, any manipulation of cheese manufacture procedures must be done carefully without affecting the artisanal cheese flavour, shape and physicochemical properties. Accordingly, the aim of this work was to understand the influence of the technological process of each cheese type on phage biocontrol activity.

The results achieved from previous determination of phage biocontrol activity in Karish, Domiati and Ras cheeses were used to calculate the phage retention rate percentages in each type using the equation in section 8.2.2.5. The results achieved showed the highest phage retention was 28.5 % in Domiati curd followed by Karish curd (22.6 %), and Ras curd recorded the lowest phage retention rate 15.0 %. The higher the phage retention rate means more phage titre remained in curd and then in cheese. A high retention rate is a critical property in antimicrobial agents, where many have argued that the extent of foodborne pathogen inactivation is dependent on the degree of agent retention in applied product (Vonasek, Le and Nitin, 2014; Ibarra-Sánchez, van Tassell and Miller, 2018; Bosch *et al.*, 2018).

The higher phage retention in Domiati curd is attributed to the unique manufacturing steps. As Domiati is rennet coagulated cheese with high salt concentration (8.0 % in milk), therefore, more whey is trapped during bonding between casein micelles producing strong gel matrix with superior water holding capacity. In addition, Domiati curd is scooped in big curd pieces in cheese cloth without cutting, which also decreases whey syneresis. However, Karish is an acid coagulated cheese, where acid is neutralizing casein outer charge that leads to casein precipitation producing weak porous micelles that have low water holding capacity (Fox, Guinee, Cogan, 2000). Although Ras cheese is rennet coagulated, curd cutting and scalding process promotes the whey to come out from casein micelles. Accordingly, the difference in cheese technological process could be linked to the variability in phage biocontrol activity in each cheese type.

From the understanding of *E. coli* phage T4 and *S. aureus* phage cocktail activity in raw milk and during raw milk Egyptian cheese treatments, the following changes in cheese processing are proposed in order to gain high phage efficacy without modifying cheese properties:

i. Inoculate selected bacteriophage in milk at the cooling storage tank before the beginning of any manufacture process, to increase time for phage adsorption and progress of bacterial infection according to target species.
ii. Delay the starter culture inoculation (if required) by a minimum of 2 h after setting the milk to the coagulation temperature.
iii. Coagulation occurs in two separate phases, firstly the primary (enzymatic) phase and secondly the secondary (nonenzymatic) phase. Given that milk gelation assembly (conversion from liquid to gel) is done in the secondary phase, we could prolong this step by adjusting the temperature at 18 °C because milk does not coagulate at or below this temperature (Fox *et al.*, 2017d).

However, the previous mentioned measures are much dependent on the cheese type and applicability in cheese manufacturing facility.

8.4 Conclusions

E. coli phage T4 was successful in decreasing inoculated and naturally present *E. coli* below the typical infectious dose after 15, 1 and 90 days in Karish, Domiati and Ras, respectively. Moreover, *S. aureus* phage cocktail (EBHT and K2) successfully reduced *S. aureus* (inoculated and indigenous) and *Staphylococcus* spp. (indigenous) below the detection limit (≤ 10 CFU/ml) after 1, 1 and 60 days in Karish, Domiati and Ras, respectively.

Those results showed the high potential of the *S. aureus* phage cocktail in particular to improve microbial safety of other raw milk cheeses with similar conditions and shows phages are an effective tool to combat *E. coli* and *S. aureus* without affecting the cheese fermentation process. Notably, the varied stability of applied phages during cheese ripening or storage raises the question of the ability of phage to fight further probable increase of pathogens due to contamination or fluctuation in storage temperature.

Some of the applied technological techniques in cheese-manufacture in the present study might be unfavourable to phage biocontrol activity, where reducing or eliminating the target pathogen in liquid milk before coagulation is a substantial step. In addition, achieved results gave a better understanding of the behaviour of phages as a biocontrol agent in different cheese types, which is necessary to develop new strategies to control pathogens in artisan cheeses.

Overall, phage-based reduction of *E. coli* and *S. aureus* was possible within a challenging high contamination level and non-target microbial population, and suggests that bacteriophages could be applied as effective biocontrol agents in Egypt and developing countries.

Chapter Nine: General Discussion & Conclusions

9.1 General Discussion

The aim of this work was to improve the safety of Egyptian raw milk cheeses by using bacteriophages without compromising the manufacture process and product quality. In order to achieve that goal, two high level research objectives were investigated: (1) Characterise selected bacteriophages and set up the ideal protocol for phage application as a biocontrol agent in dairy environment, (2) Examine the limitations and success of bacteriophages for controlling *E. coli* and *S. aureus* strains in milk and Egyptian raw milk cheeses. While the study outcomes are promising, there some points that could be explored in further research, which will be discussed in more details below.

9.1.1 Phage characterization and selection

The following phage characteristics were examined: host range, phage minimum inhibitory concentration and lytic ability under varied physicochemical conditions. Phage characterization facilitated the designing of phage application protocols in milk and raw milk cheeses (Karish, Domiati and Ras) and gave an evident vision of phage expected behaviour in cheese application. The research has provided the first information in the scientific literature of the *E. coli* and *S. aureus* phage and host strain behaviour, under wide range of pH and salt concentrations, which covers the physicochemical conditions of typical artisanal cheese (Chapter 6). To thoroughly address this objective, other goals were completed in chronological order.

Sixteen *E. coli* and twenty *S. aureus* identified strains were isolated from 100 individual cows (Chapter 4). The isolation work was performed to build a

large culture collection for testing of phage host ranges. Of note, the aim of isolation work was for the isolates to be used as host strains for phage isolation. According to literature, it is recommended to isolate phages from the same source of application, because of their prevalence, wider host specificity and greater ability to adapt in host environment which is more likely to produce a higher percentage of success (O'Flaherty, *et al.*, 2005b; García *et al.*, 2009; Porter *et al.*, 2016; Hoang *et al.*, 2016). However due to lower host count in collected milk (Chapter 4), limitation of study duration and the intended future application of this study in Egyptian environment, this phage isolation work was replaced with other objective: investigating the effect of raw milk-associated microorganisms and skimming of milk fat on selected *E. coli* and *S. aureus* phage lytic activity.

Host isolation work was necessary to determine the range of phage specificity against dairy derived *E. coli* and *S. aureus* strains from the same application source; in addition to the isolated strains, reference strains of *E. coli* and *S. aureus* were also obtained (Table 3.2). In UK, *E. coli* O157:H7 is a hazard group 3 organism, and the work with it must to be performed in a category three containment laboratory (NHS, 2015), which is not available in HAU. Accordingly, five non-pathogenic *E. coli* surrogates were obtained (Table 3.2). According to Stratakos and Grant (2018), EcoShield phage (Intralytix, USA) was able to target these non-pathogenic *E. coli* surrogates, however that phage mixture was not permitted to be used in this study.

Four *E. coli* and four *S. aureus* phages and their respective host strains (Table 3.3) were selected according to the most reported phages in literature (for *E. coli* phage T4 and *S. aureus* phage K) (O'Flaherty *et al.*, 2005a; Gill *et al.*, 2006; Liu *et al.*, 2015; Cooper, 2016) and fixed availability. Interestingly, phage host ranges testing (section 6.3.3) showed that *S. aureus* phage EBHT, K1 and K2 had a wide host specificity (89 %) against most of the reference and isolated *S. aureus* strains, and other Staphylococcal spp.; however, *E. coli* phage T4 had narrow host specificity (24 %) against reference and isolated *E. coli* strains. The wider host range of *S. aureus* EBHT and K1 could be related to their farm environmental source. This is supported by O'Flaherty *et al.* (2005b) who found that *S. aureus* phage CS1 and DW2 (isolated from farmyard) had stronger lytic

ability against *S. aureus* strains isolated from farm environment compared to clinical isolates.

In particular, determination of phage minimum inhibitory concentration initially in liquid media against different host concentration (section 6.3.1) was substantial; in contrast to the basic assumption in many studies as stated in the literature review (Chapter 2), that the higher the phage concentration the higher the biocontrol efficacy (García *et al.*, 2007; Hagens and Loessner, 2010; Gouvêa *et al.*, 2016; Yeh *et al.*, 2017). According to the obtained results, *E. coli* phage T4, *S. aureus* phage EBHT and *S. aureus* phage K2 at 10^5 PFU/ml completely inhibited host strain growth, even at high cell concentration 10^5 and 10^6 CFU/ml. Although using 10^6 PFU/ml phage concentration showed the highest lytic ability, there was no significant difference between using 10^5 and 10^6 PFU/ml. These findings revealed the minimum needed phage concentration (10^5 PFU/ml) with achieving the highest lytic ability against respective host strain (10^4 CFU/ml). This concentration was further used in phage testing under varied physicochemical conditions, which had more practical sustainability from time and cost point of view (Vipra *et al.*, 2013).

The three most popular cheeses in Egypt - Karish, Domiati and Ras - represent different categories and manufactured from raw cow's milk without using starter cultures (Hammam *et al.*, 2020). Karish is a fresh acid-coagulated cheese (Sameh, 2016); Domiati is a soft rennet-coagulated cheese, and unlike any other cheese, between 5 and 15 % salt is added directly to milk at the beginning of manufacture (Ayad, 2009); and Ras is a hard rennet-coagulated cheese (Awad, Ahmed and Soda, 2007). Therefore, the physicochemical conditions screening of Karish, Domiati and Ras during manufacture and storage (Chapter 5) gave a clear idea about the conditions that phage would be exposed to during cheese treatment. Moreover, the manufacture protocol was validated, because of the variation in cow breed, milking conditions, geography and scale of manufacture (Bittante *et al.*, 2021; Guo *et al.*, 2021) compared to that in Egypt.

The lytic ability of the four *E. coli* and four *S. aureus* phages, was tested under the estimated critical physicochemical conditions (section 5.3.5): temperature (4, 24, 37 and 45 °C), pH (4.2, 5.1, 5.9 and 6.7) and salt concentration (0.5, 4, 8, 12%) using microtitre plate assay; in addition, the *E. coli*

and *S. aureus* growth was monitored under those conditions. The lytic ability of *E. coli* and *S. aureus* phages was stable at tested temperatures and phage activity was related to host growth rate at each tested temperature (Tomat *et al.*, 2014a; Liu *et al.*, 2015). However, the phage lytic activity was not recorded at 4 °C because of inability of *E. coli* and *S. aureus* host strains to grow; Chang *et al.* (2019) reported that phage SA13m reduced *S. aureus* count by 4.1 log CFU/ml at 4 °C. Thus, it is recommended to plate the samples, where the host showed no recorded growth, to answer the question of, whether the phage is able to kill the host strain under this condition or not. At low pH (4.2), phages lytic ability was decreased, which was expected due to the recorded negative effect on phage adsorption (Tomat *et al.*, 2014a) and host growth rate (Callon *et al.*, 2016). Notably, it would be expected that Shiga toxin-producing *E. coli* would survive more at low pH (3.8 – 4.3) compared to generic *E. coli* (Haberbeck *et al.*, 2015; Callon, Arliguie and Montel, 2016). However, Duffy, Grau and Vanderlinde (2000) found high acid resistance variability between EHEC (associated with foodborne outbreaks) and generic *E. coli* strains that preadapted in same food acidic conditions.

Moreover, phages were able to inhibit recorded *S. aureus* growth at high salt concentrations (8.0 and 12.0 %), where the effect of high salt levels on *E. coli* and *S. aureus* phage activity was not tested before. Interestingly, this data is supported by the only published report by Fister *et al.* (2016b) who found no reduction in *L. monocytogenes* phage (P100) infectivity during 24 h incubation in 2 M NaCl (11.69 %) TSB solution. These data could contribute to further studies not only in artisanal cheese but also in other food products with similar physicochemical properties.

Building on presented data, *E. coli* phage T4, *S. aureus* EBHT and K2 were selected based on their high lytic ability, broad host range and efficiency under varied physicochemical conditions. Moreover, *S. aureus* EBHT and K2 were used together as a cocktail in milk and cheese application.

9.1.2 Phage biocontrol activity in dairy environment

The following goals were investigated to determine phages success for controlling *E. coli* and *S. aureus* strains in the dairy environment: a) phage biocontrol activity in sterilized (whole and skimmed) raw (whole and skimmed) milk; b) phage adsorption rate to milk components; c) phage biocontrol during

Karish, Domiati and Ras cheese manufacture and storage. The research has provided the first information in the scientific literature in: a) determination of phage MIC in unhomogenized, heat-treated (sterilization) whole and skimmed milk; b) matching phage biocontrol activity in raw whole and skimmed milk; c) determination of phage adsorption rate in sterilized (whole and skimmed) and raw (whole and skimmed) milk; d) application of *E. coli* phage in raw milk cheese; e) application of *E. coli* and *S. aureus* phages in pickled cheese.

9.1.2.1 Phage biocontrol activity in milk

Although the 10^5 PFU/ml phage concentration was able to completely inhibit the growth of host strain in liquid medium (section 6.3.1), it was anticipated that a higher phage concentration would be required for milk application to achieve the same biocontrol activity (Guenther *et al.*, 2012; Tomat *et al.*, 2018; Huang *et al.*, 2018).

The selected *E. coli* phage T4 and *S. aureus* phage cocktail (1:1 EBHT and K2) at 10^6 and 10^8 PFU/ml were successful in complete eliminating (≤ 10 CFU/ml) host strain after 6 and 2 h, respectively, in sterilized whole and skimmed milk. However, phage application in raw milk was shown to be influenced by phage host range, presence of natural microbiota and heat-labile milk component. Due to *E. coli* phage narrow host range, 4.0 ± 0.6 log CFU/ml *E. coli* was still present after 24 h in raw whole and skimmed milk. However, the observed wide host range of *S. aureus* phage cocktail achieved complete inhibition of inoculated and indigenous *S. aureus.* Therefore, it would be recommended to use a mixture of wide host range phages to achieve high pathogen elimination in raw milk.

The *E. coli* and *S. aureus* phages biocontrol activity and adsorption rate testing in sterilized (whole and skimmed) and raw (whole and skimmed) milk was coherently designed, using the proper selective media and controls. That design was shown to be effective in differentiating between the effect of tested phages on inoculated (host) and naturally present pathogen; in particular, the design gave a better understanding of the effect of cow's milk components on *E. coli* and *S. aureus* phage lytic activity. These points contributed to the identified gaps in the literature (section 2.6.3 and 7.3.3).

According to the most recent review on phage biocontrol activity in dairy environment, the exact milk component and mechanism that affect phage lytic ability in milk is still unknown (García-Anaya *et al.*, 2020). To date there are limited studies by O'Flaherty *et al.* (2005a), Gill *et al.* (2006) and García-Anaya *et al.* (2019) who focused on studying the interaction between milk components and phage. According to the analyses of obtained results and criticising those studies and other studies as discussed in section 7.3.3, it is hypothesized that IgM, IgA and milk fat are the main components that interfere with phage-bacteria interaction and consequently reduce or suppress phage antibacterial activity in raw milk.

9.1.2.2 Phage biocontrol activity in Egyptian raw milk cheeses

This is the first study on using phages as biocontrol agents against *E. coli* and *S. aureus* pathogens in Karish, Domiati and Ras cheeses. The *E. coli* and *S. aureus* phage (10^8 PFU/ml) treatments were performed under mimic conditions of Egyptian artisanal cheese of: high *E. coli* and *S. aureus* contamination level (10^4 CFU/ml), raw milk TBC (5 - 6 log CFU/ml) and validated manufacturing conditions (Chapter 5).

The inoculated and indigenous *E. coli* count increased at low curd pH of Karish (4.5) and Ras (5.9) to ~6.6 log CFU/g, in addition, detection of 5.3 and 4.3 log CFU/g *E. coli* at the end of Karish (15 days) storage and Ras (90 days) ripening, respectively. This is in accordance with previous findings that showed the ability of *E. coli* T4 host to grow and survive at 5.9, 5.1 and 4.2 pH in LB broth media during 24 h of incubation at 37 °C. In addition to the presence of 0.4 ± 0.1 % and 3.9 ± 0.1 % salt in Karish and Ras cheese, respectively, that concentration reduced *E. coli* growth rate without affecting phage lytic ability, when previously tested in broth media. However, in Domiati, *E. coli* was not able to grow in milk during coagulation time, which could be attributed to the high salt concentration in milk at beginning of manufacture. Salting in Domiati cheese is done directly in milk, gave a concentration of around 8.0 % in milk, which decreased to 4.2 % in curd after whey separation, increasing gradually again to 6.0 % after 42 days of ripening in brine solution (section 5.3.2). Likewise, *E. coli* phage T4 host showed no growth in LB broth with 8 % salt.

Notably, the expected difference between used *E. coli* and STEC *E. coli*, is that STEC *E. coli* had higher ability to survive stressful conditions during production of cheddar (Chon *et al.*, 2020), smear-ripened cheese (Maher *et al.*, 2001) and lactic cheeses (pH= 4.3) (Vernozy-Rozand *et al.*, 2005). Although using selective chromogenic media was necessary to differentiate between *E. coli* and other coliform strains present in raw milk, it is noteworthy that real *E. coli* count may be higher, due to restriction of some *E. coli* strains to grow on this media (Verhaegen *et al.*, 2015).

The *E. coli* phage T4 treatment reduced effectively inoculated and naturally present *E. coli* to 2.1, 1.6 and 2.5 log CFU/g in curd of Karish, Domiati and Ras, respectively. However, due to narrow phage T4 host range 1.3, 0.6 and 2 log CFU/g *E. coli* at the end of Karish, Domiati and Ras storage or ripening, respectively, was still present. This implies the ability of *E. coli* phage T4 to successfully decrease inoculated and naturally present *E. coli* below the typical infectious dose (FDA, 2009).

On the other hand, the inoculated and indigenous *S. aureus* count increased to 4.8 and 5.4 log CFU/g in curd of Domiati and Ras, respectively. However, due to low pH of Karish, the *S. aureus* count decreased to 3.4 in curd; in addition to detection of 3.8, 4.8 and 5.3 log CFU/g at the end of Karish (15 days) storage, Domiati (30 days) and Ras (90 days) ripening, respectively. This is in accordance with previous findings in section 6.3.2 which showed the ability of *S. aureus* EBHT host to survive with lower growth rate at 4.2 pH in LB broth media during 24 h of incubation at 37 °C. In addition, the ability of *S. aureus* to grow at high salt concentration (8.0 %) exerted during milk coagulation time, was previously observed in *S. aureus* phage EBHT testing in LB broth with 4.0 and 8.0 % salt after 24 h at 37 °C. This implies the ability of *S. aureus* strains to survive all the technological processes of Karish, Domiati and Ras cheeses. Although *S. aureus* enterotoxins production was not detected because phage host strains are non-enterotoxigenic strains, it could be assumed that presence of 10^3 to 10^5 CFU/g *S. aureus* would be sufficient for enterotoxin production, based on the upper limit for coagulase positive staphylococci in cheese (Commission Regulation [EC] No., 2073/2005).

Interestingly, the *S. aureus* phage cocktail treatment was able to completely reduce inoculated and naturally present *S. aureus* and *Staphylococcus* spp. below the detection limit (≤ 10 CFU/g) up to the end of Karish, Domiati and Ras storage or ripening, due to the high lytic ability and recorded broad host range (section 6.3.3) of selected phages.

Overall, the decline in phage titre was shown to be linked to the recorded loss of phage in whey and negative effect of low curd pH on phage survival. As, the phage ability to work and survive at low pH value was observed in previous experiments in LB broth media, this could justify the stability of phage cocktail during cheese storage. The phage stability during cheese storage raises a question about the ability of phage to fight further probable increases in pathogens due to contamination or fluctuations in storage temperature. The phages host range and phage application in raw milk cheese confirm the high specificity of used phages to target species without affecting added starter culture or natural milk microbiota, building on the fact that the unique sensorial characteristics of the cheese will not be affected. Although there is limited data on the effect of phage treatment on food organoleptic properties due to the limitations of panel test, Perera *et al.* (2015) reported that color, taste, and appearance of tested lettuce, apples, cheese, smoked salmon and frozen foods were not affected by using ListShield as biocontrol agent.

For a number of reasons, *E. coli* and *S. aureus* phages have a high potential to be used as a biocontrol agent in Egyptian environment: firstly, selected phages were not able to target the LAB strains (section 6.3.3) that were previously isolated from Egyptian raw milk cheeses and used as a starter culture in Ras cheese manufacture (Awad, Ahmed and Soda, 2007); secondly, they recorded high biocontrol activity at a challengingly high (between 5 and 6 log CFU/ml) non-target microbial population. This research shows that *E. coli* and *S. aureus* phages are a promising tool for controlling target pathogen, which are a serious problem not only in regions with hot/dry weather conditions, but also recently in European countries. According to a recent study by Feliciano *et al.* (2021), *E. coli* is one of the raising threat to raw milk in France, due to the increase in temperature driven by climate change.

9.2 Conclusions

The achieved results in the study support the hypothesis that was being tested of the ability of bacteriophages to make efficient control of foodborne pathogens in Egyptian raw milk cheeses without altering the standard manufacture process and cheese quality. In doing so, two high level research objectives were accomplished: The key conclusions which originated from research objectives are:

i. Isolation of target strain from the same raw milk used for cheese manufacture is necessary to understand the source of contamination. Achieved results showed that testing selected phages against the same raw milk isolates gave a complete understanding of the range of phage host specificity and gave efficient prediction of phage ability to target the indigenous raw milk pathogen.
ii. Determination of phage minimum inhibitory concentration initially in liquid media against different host concentration was substantial and recommended to be necessarily tested for any phage practical application in food system.
iii. Screening the physicochemical conditions during manufacture and storage of raw milk Egyptian cheeses, representing different cheese types (Karish, Domiati and Ras), was indispensable to determine the changes in physicochemical conditions and the critical pathogen control points in relation to the phage lytic ability under those conditions. Furthermore, understanding the survival of *E. coli* and *S. aureus* strains under especially low pH and high salt concentration, gave a clear expectation of their behaviour in cheese manufacture and supported the justification of the *in-situ* phage application results.
iv. According to the analyses of obtained results, it is hypothesized that IgM, IgA and milk fat are the main components that negatively affect phage biocontrol activity on raw milk. However, further studies need to be performed to understand the

mechanism of interaction between phage and milk components (IgM, IgA, fat and proteins).

v. The phage-based reduction of *E. coli* and *S. aureus* under mimic conditions of Egyptian artisanal cheese of: high *E. coli* and *S. aureus* contamination level (10^4 CFU/ml), raw milk TBC (5 -6 log CFU/ml) and manufacturing conditions, showed the high potential of phages to be applied as a biocontrol agent in those cheeses, without interfering with natural microbiota or traditional manufacture process. Of note, some recommendations for phage application in dairy environment as stated in section 7.4 and 8.3.4, would provide new insight for further studies.

9.3 Future Directions and Recommendations of Study

Egyptian raw milk cheeses especially Ras cheese have a high consumer preference in Egypt and all-over Arabian countries; however according to the latest report from the Egyptian chamber of food industries only 3750 Tons of Ras cheese was exported during the period 2018 to 2022. The main obstacle to export Ras cheese is non-matching of Ras microbiological quality to the international standards, due to the presence of pathogenic bacteria or their metabolites. This project demonstrates the potential of bacteriophage to control high *E. coli* and *S. aureus* contamination under mimic Egyptian conditions. Therefore, future studies will be planned to improve the safety of Egyptian raw milk cheeses mainly Ras cheese and develop their manufacture techniques. Improving Egyptian raw milk cheeses will expand global market in Arabian countries and Arab communities all over the world.

According to the gained data and expertise during this study, the future research will focus on main goals:

I. Phage isolation from Egyptian environmental conditions, that is recommended in order to find wide host range phages.

II. Apply *S. aureus* phage cocktail in Egyptian raw milk cheeses considering the possible post-contamination or temperature fluctuation.

III. Study the effect of isolated or commercial phages against other pathogens such as *Campylobacter* spp. and *Salmonella* spp.

IV. Cooperate with the Egyptian National Food Safety Association (NFSA), who responsible for approval of new (unregistered) food additives. Approval of phages to be used in food products will open the way for other commercial bacteriophage products to be tested and applied in raw milk cheeses or other food applications.

V. Engage with Ras cheese producers to understand the manufacture problems and application of phages in big scale.

VI. Engage with small milk producers through NFSA to increase the awareness of food safety requirements, that would be important to improve the microbiological quality of raw milk.

Another study that would be important in order to have a clear understanding of the influence of IgM, IgA, fat and proteins, further studies should be established in that area. It is recommended to stain ultra-thin section for TEM with using the primary anti-serum for cow IgG, IgM, or IgA, to visualize phage, bacterial cells, fat and immunoglobulins using Transmission Electron Microscopy (TEM). The fluorescence labelling by using confocal laser scanning microscope would also be recommended. Understanding of the mechanism of interaction between phage and milk components would have a great impact on other applications such as bovine clinical mastitis treatment.

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