Antimicrobial, Growth, Acid and Aroma Properties of the Dominant Microflora Isolated at Different Stages of Gouda Cheese Production

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DECLARATION

I declare to the best of my knowledge that this dissertation has never been submitted for any award at any institution of higher learning.

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To my beloved husband, children and mum for their continued love and support in my life.

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ABSTRACT

The microflora of Gouda cheese is dominated by starter culture lactic acid bacteria (SLAB) and non-starter organisms, which are not deliberately added. Both of these groups of microorganisms are responsible for development of the cheese flavor and organoleptic properties during ripening. This study generally aimed to characterize the dominant microbial species at different stages of Gouda cheese production in order to identify non-starter bacterial strains with positive effects for possible application as starter culture adjuncts in fermented dairy matrices.

Ten (10) independent samples from the Gouda cheese production line at Sanatos Dairies (U) Limited were collected at each of the following stages of production: raw milk, pasteurized milk, non-spiced Gouda cheese of one week, non-spiced Gouda cheese of one month, and spiced Gouda cheese of one month. The samples were enumerated within 8 h, for total viable counts (TVC), yeast and molds, total *Lactococci*, *Staphylococcus* spp., total coliforms/*E. coli* and total LAB using standard protocols. After enumeration of plates, colonies with different morphologies were purified (isolated) and examined for cell morphologies, Gram reaction and biochemical characteristics for presumptive identification and subsequently speciated the bacterial isolates using 16S rDNA sequence analysis. The dominant species were examined for genetic diversity using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering. Selected isolates from each of the major clusters were evaluated for antimicrobial activity, growth, acid and aroma production capacity in Ultra High Temperature (UHT) milk in order to prospect their possible application as starter culture adjuncts in fermented milk.

LAB (2.34 - 7.77 log CFU/g) dominated the microflora of raw milk and cheese samples. TVC in respective samples corresponded with LAB counts implying that LAB were the major organisms in different samples examined. Of the 125 microbial isolates, 121 (96.8%) were found to be bacteria and were presumptively identified as *Staphylococcus* spp. (26.5%), *Streptococcus,* or *Enterococcus* (23.9%), *Klebsiella, Enterobacter, Eschericia coli, Salmonella, Proteus, Serratia,* or *Yersinia* (19%), *Bacillus, Streptomyces, Listeria* or *Corynebacterium* (9.9%) and *Lactobacillus* or *Clostridium* were 14.1%. 16S rDNA sequence analysis delineated 28 species with *Lactococcus lactis* (12.5%), *Streptococcus lutetiensis* (8.8%) and *Lactobacillus plantarum* (5%) being the major LAB species detected in all cheese samples; the rest of the isolates were either spoilage or pathogenic bacteria that could have found their way into the cheese post production. UPGMA clustering of these organisms associated them according to their different species and source of isolation; *L. lactis* from non-spiced Gouda cheese matured for one week were clustered together with *L. lactis* from spiced Gouda cheese matured for one month implying similar genetic relatedness. Each of the *S. lutetiensis* and *L. plantarum* clusters were only obtained from spiced Gouda cheese matured for one month. Thus, *Lactococcus lactis* and *Lactobacillus plantarum* were evaluated further for growth, antimicrobial activity against selected pathogenic bacterial strains, acid and aroma production properties.

Single and mixed cultures of these organisms had similar growth patterns. All isolates inhibited *E. coli*, *Salmonella* and *S. aureus* on solid media assays but only one isolate of *Lc. lactis* (Gw-05) inhibited *E. coli* and *Salmonella* in the cell-free supernatant assay. In single culture, *Lc. lactis* had better acid production properties than *Lb. plantarum*. Mixed of these organisms enhanced acid production. During short term ripening, *L. plantarum* synthesized more organic acids, ketones, esters and aldehydes (13.7%, 2.3%, 9.3%, and 0.8%, respectively) than *L. lactis* which mostly produced organic acids and esters (38.7%, 6.6%, respectively). Co-culture of these organisms inhibited aroma production; only three organic acids and one ester were produced. During long term ripening, each of *L. lactis* and *L. plantarum* only produced organic acids and esters. However, *L. plantarum* produced more esters than *L. lactis*. Co-culture of the organisms enhanced aroma production resulting from synthesis of organic acids, esters and one ketone.

It was therefore concluded that LAB dominate the microflora of Gouda cheese production. *L. lactis* has better prospects for application as primary starter culture in dairy products because they promote rapid acidification of milk, which is desirable for curd formation. Short-term fermentation of milk by single culture of *L. lactis* and *L. plantarum* enhance aroma production, while long term ripening enhances aroma production in mixed culture. Single cultures of these organisms could therefore enhance aroma formation in yoghurt, which requires short-term fermentation, whereas mixed culture could be applied in cheese, which requires long-term ripening.

CHAPTER ONE: INTRODUCTION

1. Background

The global annual cheese production stands at 22.17 million metric tons (Statista, 2023). Cheese is mainly produced and consumed by Europeans in Europe and in countries such as Egypt and South Africa with consumption rates of 10.1 kg and 1.0 kg per capita, respectively (IDF, 2012). Cheese production and consumption statistics for Africa and Uganda is scanty . However, 3500 liters of milk are used daily for cheese production in Uganda mainly by Gouda Gold and Paramount Dairies Ltd (Dairy Development Authority, DDA, 2009). Gouda cheese is the most common (60%) ripened cheese worldwide as well as in Uganda (Walstra et al., 1993; Kjær, Muhumuza and Mwebaze, 2012). Gouda originated from the Netherlands as a washed-curd cheese produced from pasteurized whole milk of bovine origin (van den Berg et al., 2004; Codex Alimentarius, 2013; Jo, Benoist, Ameerally and Drake, 2018). The curd is usually salted and subjected to ripening for one month to three years at 13-15^{o}C (Mellgren, 2005; Jung et al., 2013). Milk acidification is obtained by *Leuconostoc mesenteroides* sub-spicies *cremoris*, *Lactococcus lactis* sub-species *lactis biovar diacetylactis* and *Lc. lactis* sub-species *cremoris* (Ruyssen et al., 2013).

Gouda cheese is smooth in texture and is yellow-orange in colour when young, or reddish when mature. Recent studies indicated that consumers mainly preferred flavor and texture of cheese (Murray and Delahunty, 2000; Young et al., 2004; Drake et al., 2008; Van Leuven et al., 2008; Childs and Drake, 2009; Shepard et al., 2013; Inagaki et al., 2015). The texture and flavor of cheese are associated with chemical properties such as fatty acids, lactones and organic acids, which are dependent on the particular microbial strains used during ripening (Califano and Bevilacqua, 2000; Alewijn et al., 2005; Inagaki et al., 2015).

Gouda cheese microbiota is mainly composed of starter culture lactic acid bacteria (SLAB) and non-starter organisms, which are not deliberately added during the production process but are introduced as incidental contaminants from raw materials and the environment. The SLAB mainly produce lactic acid and are involved in ripening of the cheese (Settanni and Moschetti, 2010). Several studies suggested that in addition to SLAB, non-starter LAB also have a role in enhancing desirable flavors and organoleptic properties of ripened cheese (Kongo et al., 2009; Aljewicz et

al., 2010; Bockelmann, 2010; Settanni and Moschetti, 2010). SLAB populations are high (about 10^8–10^9 CFU/g) at onset of ripening however, these generally decrease as the cheese ages (Zago et al., 2007; Franciosi et al., 2008). In comparison, the non-starter LAB have a potential of increasing in population from less than 10 CFU/g to approximately 4 to 5 log CFU/g during the first few weeks of ripening (Briggiler-Marco et al., 2007; Kongo et al., 2009). Also, yeast and/or bacterial ripening starter culture organisms normally colonize the matrix of the cheese while the non-starter flora contaminants from the factory premises usually are ones which dominate the cheese surface (Brennan et al., 2002; Petersen et al., 2002; Feurer et al., 2004).

The non-starter LAB during metabolism promotes flavor development while the cheese undergoes ripening. However, different strains may have profound effects on product flavor, texture and other properties leading to inconsistences in product sensory profiles (Settanni and Moschetti, 2010). This study therefore characterized the dominant microbial species at the different stages of Gouda cheese production in order to identify strains with positive effects for possible application as starter culture adjuncts in fermented dairy matrices with consistent quality attributes.

1.2 Problem statement

The cheese fragment of the dairy industry in Uganda is estimated at an annual growth rate of 9.5%, fetching a revenue of USD 71 million annually regardless of the low domestic per capital consumption (CAGR, 2023). Concerns related to the quality and safety of the cheese such as incompetence in the production process, lack of consumer awareness about its nutritional benefits and its relatively higher cost could be responsible for its low consumption in Uganda (Fox, Guinee, Cogan, and McSweeney, 2017). The source of these quality and safety concerns of Gouda cheese produced in Uganda is unknown. Fromageries et al., (2008), Gkatzionis et al., (2009), and Murtaza et al., (2013) attributed variations in cheese quality to changes in the metabolic activities of its inherent microflora, which comprises starter cultures and non-starter microbial contaminants. These variations also result in poor prediction of cheese quality from batch to batch, resulting in product rejection by consumers. Aside from the recognized added starter cultures, the adventitious non-starter microorganisms that potentially infiltrate the Gouda cheese production process at various phases for Ugandan variants have not been well investigated. Therefore, this study aimed at identifying and characterizing the dominant microbial populations at various stages of Gouda

cheese production in Uganda in order to investigate their possible impact to product quality. Microbial strains that could improve cheese quality properties were further evaluated for potential adoption as starter culture adjuncts in fermented dairy products.

1.3 Justification

Among dairy products, cheese is commonly consumed in Uganda after ghee, yoghurt, butter and ice cream in that order, due to its nutritional benefits and desirable sensory characteristics (Aliguma and Nyoro, 2016). Owing to the variability in microbial populations and strain composition, non-starter microorganisms can lead to inconsistencies in cheese quality and flavor (Fromageries et al., 2008; Gkatzionis et al., 2009; Murtaza et al., 2013; Mugampoza et al., 2020). Several studies reported marked variations in the proportions of strains and species dominating cheeses even when they are produced under the same processing environments (Fromageries et al., 2008; Gkatzionis et al., 2009; Bove, et al., 2011; Murtaza et al., 2013). Moreover, succession of microbial spp. and strains may lead to domination of non-starter microbiota in cheese at different ripening stages (Settanni and Moschetti, 2010), which may bring about product quality inconsistences since their occurrence and concentration in cheese is not regulated in the production process. Establishing the dominant non-starter microorganisms that could have positive impact on the sensory attributes of Gouda cheese, and probing their possible use as starter culture adjuncts is intended to provide a bench mark for minimizing the problem of quality inconsistencies in cheese manufacture.

1.4 Significance

Findings from this study will inform cheese processers about the dominant microbial populations at different stages during production of ripened cheese in Uganda. Understanding the dominant microbial populations (LAB and NSLAB) provides information to examine their potential contribution to sensory and chemical properties of Gouda cheeses so that processers would develop ripened cheese with consistent acceptable sensory attributes. In addition, the strains responsible for the distinctive desirable flavor profiles were isolated, identified and prospected for commercial application in production of fermented milk.

1.5 Objectives

1.5.1 Main objective

To assess the technological properties of the dominant microflora during Gouda cheese production for its controlled quality production.

1.5.2 Specific objectives

1. To establish diversity of microorganisms which occur at different stages of Gouda cheese production.

2. To assess the antimicrobial activity of the isolated dominant lactic acid bacteria against indicator microorganisms.

3. To examine the growth, acid and aroma production capacity of the dominant lactic acid bacterial species isolated at the different stages of Gouda cheese production.

1.6 Hypotheses

1. There is no significant difference in the composition of microbial species, which occur at different stages of Gouda cheese production.
2. There is no significant difference in antimicrobial properties of the dominant microbial species, which occur at different stages of Gouda cheese production.
3. There is no significant difference in growth, acid and aroma production capacity of the dominant microbial species, which occur at different stages of Gouda cheese production in the milk model medium.

CHAPTER TWO: LITERATURE REVIEW

2.1 Definition and classification of cheese

Cheese is a fermented dairy product with medium moisture content of 30-50%, and with an extended shelf life of more than six months (Ramesh, Chandan and Kooper, 2011). The process of making Cheese leads to partial dehydration of the product due to removal of whey from the curd. When salt is added, antimicrobial metabolites are generated due to microbial activity (Al-Nabulsi et al., 2020). The ripening process produces low molecular weight water-soluble compounds and lactic acid which lowers the pH. These compounds preserve the cheese making it safe for human consumption (Singh and Cadwallader, 2008). Cheese is concentrated in nutrients and comes with a variety of texture and flavors. Gouda cheese is sweet, with limited number of eyes, and a semi hard texture shaped in form of a wheel sized 3 to 20 kg (Wong, 1974; Zall, 1992; Kosikowski and Mistry, 1997).

There are different classification schemes for cheese to avail nutritional information and facilitate international trade since there are over 100 cheese varieties in the world (Sandine and Elliker, 1970). Cheese can be classified into eight categories according to firmness and moisture content (Table 1) (Walter and Hargrove, 1972). High moisture content is correlated with soft texture and shorter shelf-life of cheese. Thus, cream and cottage cheese which have very high moisture content, are not aged. Other categories include very hard, hard cheese with and without eyes, semisoft and soft cheeses (Table 1).

Table 1. Classification of cheese according to firmness

Cheese category	Ripening microorganisms	Example
Very hard	Bacteria	Parmesan, Romano
Hard	Bacteria	Cheddar, Granular
	Bacteria	Emmental, Gruyere
	Bacteria	Brick, Munster
Semisoft	Bacteria and surface microorganisms	Limburger, Gouda
	Blue mold in the interior	Stilton, Danablue
Soft	Ripened	Camembert, Brie
	Unripened	Cottage, Pot

Source: Walter and Hargrove (1972)

2.2 Cheese production and consumption

The global annual cheese production is estimated at 22.17 million metric tons (Statista, 2023). In Uganda, annual cheese production stands at 9.52 million kilograms (Statista, 2023). The aim of cheese production is to preserve the wholesomeness of milk by fermentation during its prolonged storage and utilization (Beresford et al., 2001). Cheese is widely consumed in Western countries as a snack, as part of a dish or as a prepackaged convenience food (Farkye, 2004). Its consumption could be due to its good flavor and a smooth mouth feel. There is scant information on per capita cheese consumption among African countries including Uganda. However, cheese consumption in Africa is generally low mainly due to its astringent/sour taste which underscores its sensory acceptability (Nyamakwere et al., 2021). Cheese is among fairly consumed dairy products in Uganda after ghee, yoghurt, butter and ice cream due to its nutritional benefits and desirable sensory characteristics (Aliguma and Nyoro, 2016). The relatively low cheese consumption in Uganda could be explained by its astringent/sour taste, which also varies from brand to brand and among the different producers (Waiswa, 2021).

2.3 Principles of cheese production

The main principle behind formation of curd from milk during cheese making is destabilization of casein to enhance gel formation which when cut, synereses; this is accelerated by mechanical stirring and mild heating (Fox and McSweeney, 2004). The process yields curd that is readily recovered from whey (Kelly, Huppertz and Sheehan, 2008). While these physico-chemical reactions are taking place, the added lactic acid bacterial starter cultures with rennet proliferate and metabolize lactose sugar to lactic acid (Fox and McSweeney, 2006). This lowers curd pH and consequently enhances cheese flavor, through production of catabolic enzymes that hydrolyze the milk composition to form several flavor and aroma compounds (Kelly, Huppertz and Sheehan, 2008). After separation of whey, the curd is pressed into a solid visco-elastic mass that is stored under conditions which allow biochemical reactions catalyzed by cheese-borne microflora, which principally consist of LAB starter cultures, non-starter microorganisms and yeasts and moulds in some varieties, which are added intentionally (Fox and McSweeney, 2004; Kelly, Huppertz and Sheehan, 2008). During ripening, inherent enzymes break down casein leading to changes in cheese texture (Fox and McSweeney, 2006).

2.4 Description and production of Gouda cheese

Gouda cheese is a wheel-shaped matured firm or semi-hard cheese with flat sides. The cheese generally has reddish or orange-waxed color; it has a firm texture and a few gas holes (Jung, Ko and Kwak, 2013). The gas holes are produced by the starter cultures during fermentation to produce the final product with 43% average moisture content and fat content of 49%, on a dry matter basis (Moneim et al., 2018).

Kosikowski and Mistry (1997) provided detailed Gouda production process as follows. Gouda is described as a sweet and renneted cheese made from pasteurized cow's milk which is partially skimmed or whole. The milk is heated to 32 - 33^{o}C followed by addition of a direct vat set mesophilic LAB starter culture. Then, 23 ml of rennet is added after 90 min and mixed for 3 min. The milk is allowed to set for 45 min at 32-33^{o}C. The curd is cut in small sizes, gradually stirred and the temperature increased to 37^{o}C for 30 min. Then, whey (10% of total volume) is recovered and substituted by same volume of water at 37^{o}C to lower lactose content and control lactic acid production during the production process. The curd-whey mixture is stirred for 30 min at the

cooking temperature, and drained of the whey. After draining, the curd is transferred to a vat for pre-press. The pre-press is done for 30 min at 1.5 bar. The curd is put into a wheel-shaped plastic mould and pressed for 20 h at 3 Bar and 2-3 h intervals. In the next step, fresh cheese is immersed in brine (20% (w/w) for 8 h at 14^{o}C. Brine is drained-off, the cheese is dried, and ripened at 14^{o}C for 1 to 6 months at 85% humidity.

2.5 Microbial contamination during cheese production

Cheese microflora mainly comprises two major groups; (i) starter lactic acid bacteria (SLAB) and (ii) secondary microorganisms. Mixed or single strains of *Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris, Leuconostoc mesenteroides, Streptococcus thermophilus, Lactobacillus helveticus* and *Lactobacillus delbruecki* are applied as SLAB mesophilic and thermophilic cultures in cheese production (Hoier et al., 2010). SLAB produce acid and also promote ripening (Settanni and Moschetti, 2010). Secondary microflora mainly consists of yeasts and non-starter lactic acid bacteria (NSLAB). NSLAB are unique to a cheese variety. NSLAB do not grow well in milk (Cogan et al., 2007; Briggiler-Marco et al., 2007) and thus do not contribute to acid production. However, they can grow during ripening stage (Agarwal et al., 2006; Kongo et al., 2009; Ciprovica and Mikelsone, 2011; Barakat et al., 2011). Therefore, an increase in NSLAB populations is typical of most cheese varieties.

Several studies reported that NSLAB can contribute to desirable flavor development and to sensory characteristics of ripened cheese (Kongo et al., 2009; Aljewicz et al., 2010; Bockelmann, 2010; Settanni and Moschetti, 2010). NSLAB can also cause excessive carbon dioxide production and development of off flavors in cheeses (Kongo et al., 2009; Settanni and Moschetti, 2010). NSLAB widely vary and mainly consist of facultative or obligatory heterofermentative lactobacilli thst belong to *L. casei, L. plantarum, L. paraplantarum; L. pentosus, L. brevis, L. curvatus, L. fermentum, L. buchneri, L. parabuchneri, L. coryniformis* and *L. rhamnosus* (Samelis et al., 2010; Ghotbi et al., 2011; Jokovic et al., 2011; Mlalazi et al., 2011; Morales et al., 2011). Amongst LAB, *Pediococcus* and *Leuconostoc* also occur albeit at a lesser level (Randazzo et al., 2009; Colombo et al., 2010; Jokovic et al., 2011). Populations of SLAB are high (about 10^{8}–10^{9} CFU/g) at the start of cheese ripening and decrease gradually during maturation (Zago et al., 2007; Franciosi et al.,

2008). In comparison, NSLAB increase by 4-5 log CFU/g within few weeks of ripening, from less than 10 CFU/g in fresh curd (Briggiler-Marco et al., 2007; Cogan at al., 2007; Kongo et al., 2009).

2.5.1 Sources of microbial contamination during cheese production

Figure 1 shows a summary of the possible microbiological contamination steps during Gouda cheese production. The 'old young' smearing technique, in which fresh cheese is washed with microbial suspension from brine derived from mature cheese, is one of the sources of contamination of cheese during production. Despite the fact that it is a reliable source of desirable organisms that enhance ripening, 'old young' smearing can also transfer pathogenic and spoilage organisms for example *Listeria monocytogenes* (Valde´s-Stauber et al., 1997). Studies reported that bacterial and/or yeast ripening starters may not develop on cheese surface but instead, adventitious microflora that originate from environment, could dominate the cheese surface (Brennan et al., 2002; Petersen et al., 2002; Feurer et al., 2004). Brennan et al. (2002), used polyphasic approach to demonstrate that *Brevibacterium aurantiacum*, despite being added intentionally on surface of Gubbeen cheese, is not mainly found among its surface flora and no bacterial succession occurs during its ripening. Mounier et al. (2005) also reported that brines are a major source of *Staphylococcus saprophyticus* and *Debaromyces hansenii* and, in addition, workers' hands are sources of *Corynebacterium casei* and *C. variabile*. Single clones of *Microbacterium gubbeenense*, *C. casei* and *Corynebacterium mooreparkense* dominate cheese surface microflora during ripening (Brennan et al., 2002). *Corynebacterium mooreparkense* is the synonym for *Corynebacterium variabile* (Gelsomino et al., 2005).

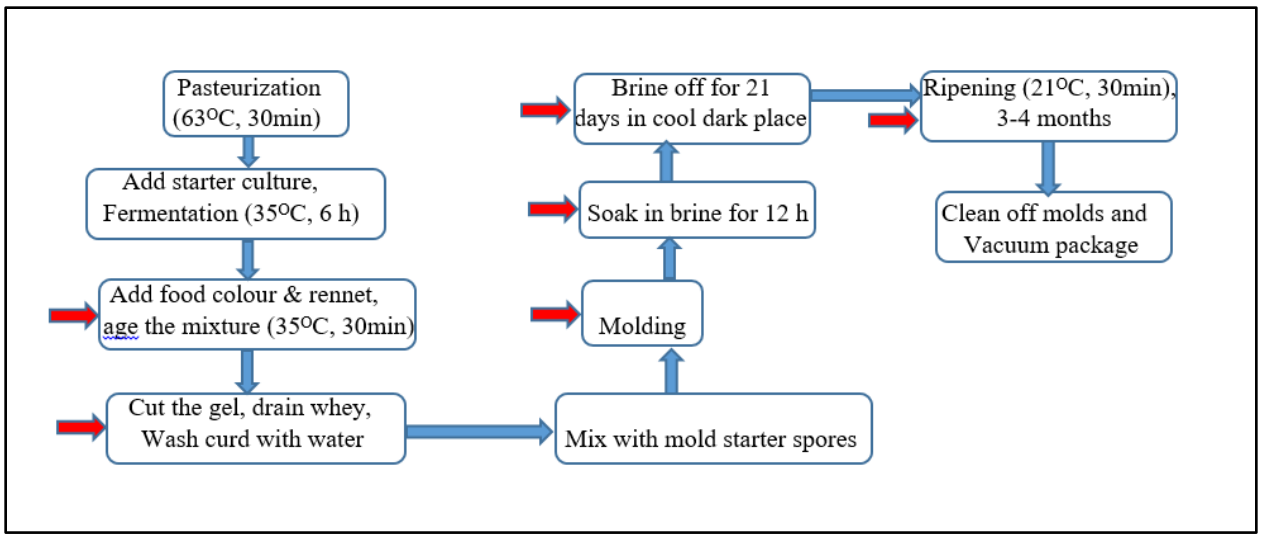

Figure 1. Production of Gouda cheese and possible contamination steps (Source: Decadt and De Vuyst, 2023)

Feurer et al. (2004) reported that, *Brevibacterium linens* that is inoculated on surface of a French smear ripened cheese in first stages of ripening, nearly disappears from the surface after three weeks of ripening. After 31 days, *Arthrobacter* dominates its bacterial flora. Other species including *Arthrobacter bergerei*, *Staphylococcus succinus* ssp. *Casei*, *Staphylococcus equorum* ssp. *linens* (Place et al., 2002, 2003) and *Arthrobacter arilaitensis* (Irlinger et al., 2005) dominate smear-ripened cheeses. *Brevibacterium linens* that was originally detected in Camembert cheese, is a member of a novel species *B. aurantiacum* (Gavrish et al., 2004) and *B. helvolum* of a novel genus *Pseudoclavibacter helvolus* (Manaia et al., 2004). On the other hand, there is less diversity in dominant yeast on surface of smear-ripened cheeses such as Gouda. *Geotrichum candidum* and *Debaryomyces hansenii* have been detected in rennet cheeses; and *Pichia membranifaciens* and *Kluyveromyces marxianus* in acid-curd cheeses (Eliskases-Lechner and Ginzinger, 1995; Petersen et al., 2002; Valde´s-Stauber et al., 1997). Petersen et al. (2002) reported that proliferation of *D. hansenii* occurs during ripening of Danbo-type surface ripened cheeses.

2.6 Microflora of Gouda cheese

Kołakowski, Podolak and Kowalska (2005) while evaluating the changes in microflora of SLAB, NSLAB and yeast in Gouda cheese reported that SLAB are the dominant microorganisms after brine application and account for 90% of the total microflora of Gouda. These authors further

reported that ripening leads to domination of yeasts and NSLAB, with concomitant decrease in SLAB.

Kołakowski, Podolak and Kowalska (2005) further reported that vancomycin-tolerant *Lactobacillus* species constitute the main NSLAB in ripened Gouda. Yeasts, which are initially low at 4 $\log_{10}$ CFU/g, increase by 2 log CFU/g after 28 days and slightly reduce at 8 and 12 weeks of ripening. Thereafter, non-starter *Lactobacillus* species exceed 90% of the total LAB populations, whereas yeast populations comprise more than 40% of the total microbial load. Thus, majority of the NSLAB consist of vancomycin-tolerant homo- and heterofermentative *Lactobacillus* spp. The three most dominant bacteria and yeast in ripened Gouda cheese are *Corynebacterium variabile*, *Corynebacterium casei*, & *Staphylococcus saprophyticus*, and *Debaryomyces hansenii*, respectively (Mounier et al., 2005). Moneim et al. (2018) reported counts of *Streptococcus*, yeast and moulds as $1.4x10^7$ CFU/g and $2x10^5$ CFU/g, respectively and suggested that there are no harmful microorganisms in ripened Gouda cheese.

2.7 Starter cultures for cheese production

Lactic acid bacterial starter cultures are used for cheese production (Hill, 2009). A starter culture imparts predictable quality attributes, which can be consistently produced over different batches of production, and it has no health concerns associated with the consumption of food in which it is contained. Cheese starters are usually non-motile, Gram positive, non-sporing, nitrite and catalase negative bacteria that are microaerophilic in nature and inactivated during refrigeration temperatures (Ramesh, Chandan and Kooper, 2011). Depending on the cheese variety, starter cultures comprise of mesophilic strains of *L. lactis* sub-species *lactis, L. lactis* sub-species *cremoris, Leuconostoc lactis* sub-species *cremoris* and *Lactobacillus lactis* sub-species *cremoris* or thermophilic strains of *Streptococcus thermophillus, Lactobacillus delbrueckii* ssp. *bulgaricus* (Andren, 2003). Both groups are discriminated based on their optimum growth temperature, which is ranges from 25ºC to 35ºC for mesophilic cultures and 39ºC to 50ºC for thermophilic ones.

2.8 Quality attributes of Gouda cheese

Gouda cheese consumption is majorly determined by the perception of the consumers (IDF, 2001). The chemical composition of the cheese determines its quality properties including texture, flavor

and color, which are progressively developed during ripening (Vasterdis, 1989). However, the quality attributes specific cheese variety and within batches are influenced by milk type, starter culture and production procedure (El-Nimr et al., 2010).

Cheese quality is assessed by the degree of its maturity, texture of the cheese and a series of standard sensory analyses to establish the absences of certain sensory characteristics due to microbial contamination during the cheese making process (Pinho et al., 2004; El-Nimr, et al., 2010). Color is a crucial quality attributes since it provides an enticing appearance to the cheese (Lebecque et al., 2001). Depending on consumer preference and intended use, the colour of cheese varies from pale yellow to deep a red-orange (El-Nimr, et al., 2010). Texture and color are also internationally associated with the country of origin of the cheese hence influencing the consumer purchasing decisions especially for the Protected Designation of Origin (Bugaud et al., 2001). However, inconsistences in the quality attributes of cheese is still challenge in the cheese making industry. This therefore call for a need to characterize the different cheeses and an in-depth study about the changes that occur during cheese ripening and intra-varietal and batch-to-batch comparisons (Lebecque et al., 2001).

2.9 Microbiological analysis of cheese

For microbiological analysis, 1 ml of pasteurized milk and 1 g of cheese are usually mixed in 9 ml of diluent in a stomacher blender. Then, 10-fold dilutions are prepared in the diluent (Van Hoorde et al., 2010). The resultant samples are used for further analysis.

2.9.1 Conventional methods

After culturing on media, Gram staining is performed to examine Gram reaction, cell shapes and arrangement of the cells of the different microbial isolates under a light microscope at X1000 (Mugampoza et al., 2020). Oxidase identification strips are used to test for production of cytochrome oxidase enzyme. Antimicrobial, proteolytic and lipolytic activities of the cheese inherent microflora are determined by development of a halo surrounding colonies on plates smeared with indicator test strains, casein hydrolysate agar and tributyrin agar plates, respectively (Van Hoorde et al., 2010; Mugampoza et al., 2020). Starter and non-starter LAB cultures are mainly responsible for antimicrobial, proteolytic and lipolytic activities, and hence enhance flavor

formation by provision of substrates for secondary metabolism (Smit et al., 2005). As the starter cultures grow, they metabolize cheese constituents mainly producing lactic acid which leads to cheese acidification (Van Hoorde et al., 2010).

2.9.2 Molecular methods

Pure culture are normally extracted for deoxyribose nucleic acids (DNA) using the cetyl trimethyl ammonium bromide (CTAB) protocol as described by Pitcher et al. (1989), Masco et al. (2003) and Mugampoza et al. (2020). The quality and purity of DNA extract are determined spectrophotometrically and visualized under UV after electrophoresis on agarose gels.

2.9.2.1 DNA extractions methods

There is need to accurately establish milk and cheese bacterial flora while understanding the key determinants of the quality attributes in dairy products. For accurate determinations, DNA-based technologies are used to assess the microbial communities in cheese. However, the accuracy of these methods are dependent on the efficiency and effectiveness of extracting reasonable amounts of pure DNA. Some of the methods commonly used for DNA extraction from milk and cheese include Modified QIAamp® DNA stool mini kit, Chemagic Food Basic kit and Wizard® Magnetic DNA isolation kit (Table 2). These methods rely on kits for DNA extraction from samples following manufacturers' instructions.

Guanidine thiocyanate protocol (Duthoit et al., 2003) is designated as the lytic method of DNA extraction and combines methods of O'Mahony and Hill (2004), Parayre et al. (2007) and Dolci et al. (2008). The DNA pellet from 1 ml milk or 1 ml homogenized cheese is suspended in 0.5 ml of breaking buffer (20 mmol/L Tris HCl, 2 mmol/L EDTA, 2% Triton X100, 50/µg/L lysozyme, 100 U mutanolysin) and kept for 1 h at 37°C for enzymatic lysis. Then, 250 µg/ml proteinase K is added and incubated for 1 h at 55°C for proteolysis. The suspension is put in a 2 ml tube containing 0.3 g zirconium beads, shaken twice for 90 s in a bead beater, and spinned for 10 min at 12000 rpm. The supernatant is put in a fresh tube and mixed gently with an equal volume of phenol: chloroform: isoamylalcohol (25:24:1) and spinned for 2 min at 12000 rpm. The top phase is put in a clean tube, 0.1% the volume of 3 mol/L sodium acetate and 2 volumes of cold ethanol are added gently mixed and stored for 24 h at -20°C. The suspension is spun for 10 min at

14000 g, the supernatant is decanted, the pellets are washed using 70% cold ethanol, they are spinned for 5 min at 12000 g, dried in an oven and re-suspended in 0.1 ml TE buffer.

Table 2. DNA extraction protocols

Method	Extraction type	Source	Principle
Modified QIAamp® DNA stool mini kit	Solid-phase/column extraction	Commercial	Silica-gel membrane used to purify DNA.
Wizard® Magnetic DNA Isolation kit	Mobile solid-phase/magnetic bead extraction	Commercial	Magnetic beads used to bind and purify DNA.
Milk Bacterial DNA Isolation kit	Solid-phase/column extraction	Commercial	Resin separation matrix and spin column chromatography used for DNA binding and purification.
PowerFood™ Microbial DNA Isolation kit	Solid-phase/column extraction	Commercial	Silica membrane spin column used to bind DNA.
Lytic method	Liquid–liquid extraction	Non-commercial	Phenol-chloroform and ethanol used for DNA extraction and purification.
Guanidine thiocyanate method	Liquid–liquid extraction	Non-commercial	Phenol-chloroform and ethanol used for DNA extraction and purification.

Source: Duthoit et al. (2003)

2.9.2.2 Molecular methods for probing genetic diversity of microorganisms of food origin

Bacterial taxonomy based on 16S ribosomal RNA sequencing analysis showed that some taxa obtained based on phenotypic characteristics do not match with their phylogenetic relatedness (Mohania et al., 2008). Phenotypic methods are also limited by their low reproducibility, being ambiguous due to plasticity of some bacterial growth, excessive logistics required for large-scale analyses, and their low discrimination (Mohania et al., 2008). Thus, genetic typing methods are often used for speciation of organisms and for strain differentiation. The main benefits of DNA-based typing protocols is their high discriminatory power and universal applicability (Farber, 1996).

Molecular techniques such as pulse-field gel electrophoresis (PFGE), rep-PCR fingerprinting and restriction fragment length polymorphism (RFLP) are highly specific and often used for characterization and detection of bacterial strains (Holzapfel et al., 2001). However, the reproducibility of rep-PCR fingerprinting is sometimes low and the method requires carefully controlled conditions. Some researchers prefer randomly amplified polymorphic DNA (RAPD) PCR for identification and characterization of bacterial strains from different sources such as food, human and milk (Kwon, 2000; Spano, Beneduce, Tarantino, Zapparoli and Massa, 2002). PFGE differentiates microorganisms of the same species, groups strains within a species, distinguishes strains that belong to different species, and places isolates in specific microbial species (Klein, Pack, Bonaparte and Reuter, 1998; Giraffa, and Neviani, 2000). Temperature gradient gel electrophoresis (TGGE) and denaturing gradient gel electrophoresis (DGGE) analysis of bacterial 16S rDNA and rRNA are powerful tools that are used to monitor and determine bacterial communities (Zoetendal, Akkermans and De Vos, 1998). However, there is wide variation in bacterial microbiota of different matrices, therefore these techniques have limitation in providing detailed analysis of multiple matrices (Mohania et al., 2008).

2.9.2.3 16S and 18S rDNA PCR sequence analysis

DNA extract is used for polymerase chain amplification of 16S rRNA (V3 region) using universal 16S primers which bind to 95% of the 16S gene. The forward primer (5′-AYTGGGYDTAAAG NG) in combination with four reverse primers (5′-TACCRGGGTHTCTAATCC), (5′-TACCAG AGTATCTAATTC), (5′-CTACDSRGGTMTCTAATC) and (5′-TACNVGGGTATCTAATC) (http://pyro.cme.msu.edu/pyro/help.jsp). The reaction mix contains 25 µl Green Master Mix, 10 pmol each primer, 5 µl DNA and sterile deionized water (final, 50 µl). PCR is performed in a thermal cycler with a programme consisting of initial denaturation at 94°C, 2 min, followed by 40 cycles; denaturation at 94°C, 1 min, annealing at 52°C, 1 min and extension at 72°C, 1 minute. Final extension is done at 72°C, 2 min. The PCR amplicon is purified and quantified using a Nanodrop. Quantity and quality are also visually examined under UV after gel electrophoresis.

2.10 Metabolic pathways for cheese flavor development

Cheese ripening begins with proliferation of yeasts that metabolizes lactic acid to carbon dioxide and water to form metabolites such as ammonia (Corsetti et al., 2001), which deacidifies cheese surface and enables growth of salt-tolerant, less acid-tolerant, catalase-positive, Gram-positive bacteria such as Coryneforms and *Staphylococcus* spp. (Kieronczyk et al., 2003). Flavor development during cheese ripening arises from several complex chemical and biochemical processes that convert milk components (Smit et al., 2005). Basically, typical cheese flavor is a result of three main processes including proteolysis of milk caseins, lipolysis and fermentation of residual lactose, lactic and citric acid which collectively lead for formation of a complex mixture of non-volatile flavor and volatile aroma compounds (Smit et al., 2005).

In addition to enzyme action from rennet and milk, microbial enzymes are also responsible for cheese flavor development during cheese ripening (Marilley and Casey, 2004). For Gouda-type cheeses, microorganisms that participate in flavor formation are categorized in two major groups, i.e., starter and non-starter lactic acid bacteria (NSLAB). Whereas starter cultures mainly produce lactic acid, they also contribute to lipolysis and proteolysis and therefore flavor formation (Smit et al., 2005). NSLAB, which may enter the cheese making process through milk, equipment and production environment, and gradually dominate cheese microbiota during ripening to reach numbers up to 10^7–10^8 cfu/g also pose an important effect in development of sensory properties of matured cheeses as a result of protein and lipid catabolism (Fox et al., 1998). Because majority of LAB produce various peptidases, proteinases and amino acid-hydrolyzing enzymes, proteolytic pathway is by far the most important process during cheese flavor development (McSweeney, 2004). In comparison, moderate lipolysis occurs in Gouda and other cheeses (Collins et al., 2003; McSweeney, 2004) largely due to limited lipolytic activity of most LAB (Broadbent and Steele, 2005). During lipolysis, the ester bonds in the triglycerides are broken down to butyric acid, which enhances the sensory attributes of the cheese (Van Hoorde et al., 2010).

2.10.1 Aroma properties of cheese

Cheese flavor is due to complex mixture of substances such as lactones, carbonyls, fatty acids (FFA), esters, alcohols and hydrocarbons (Achaya, 1997). These compounds are mainly derived from microbial metabolism of various milk constituents (Sserunjogi et al., 1998). Production of

cheese flavors mainly depends on *Lactococcus lactis* ssp. *cremoris* and *lactis*, which are blended as starter cultures for cheese making (Banks, 2003). The acid production capability of these organisms is optimum at 30^{o}C to 35^{o}C with the *lactis* subspecies mainly essential for acid development while the *cremoris* subspecies play and incremental role in flavor development. The variant species *diacetylactis* produces carbon dioxide that causes perforations in the cheese and diacetyl which is responsible for a buttery flavor from citrate. The weak acid producing starter culture *Leuconostoc mesenteroides* sub-species *cremoris* also forms diacetyl and carbon dioxide. Diacetyl, a flavor compound, is important in fresh cheese production. Thus, the above listed starter organisms are often used in soft ripened, Cheddar & Gouda, most fresh and washed cheese varieties (Barbano, 2001). Thermophilic starter cultures are mainly used in Gruyere, Swiss and some Italian cheeses like Mozzarella. In addition to lactate, thermophilic cultures generally produce acetaldehyde.

2.10.2 Flavor analysis of food

Characterization of flavor compounds at molecular level is important for determination of the chemical nature and impact of these compounds on food quality (Fu et al., 2016; Yu, Duan, Jiang and Hao, 2017). Conventional protocols for molecular examination of organic compounds normally requires extraction of the sample collected on a filter into solvents followed by evaporation, separation and detection by an instrument such as a gas chromatography mass spectrometry (Noziere et al., 2017). However, solvent extraction methods are susceptible to contamination and the analyte may be lost during sample pre-treatment. In addition, they may pollute the environment due to use of a large quantity of solvents (Urban, Alves, Allen, Cardoso, Queiroz and Campos, 2014; Yang, Hsu, Chen, Young, Huang and Ku, 2017). Solvent extraction methods are also usually time consuming. Thus, thermal desorption is normally taken as an alternative procedure and uses high temperature to evaporate organic analytes from the filter for subsequent GC-MS detection (Chow et al., 2007; Xu et al., 2013). Never-the-less, both thermal desorption and solvent extraction methods are sensitive to low concentration of aromatic compounds and have good recovery, repeatability and reproducibility (Mohania et al., 2008).

Compared to solvent extraction, thermal desorption (TD) is convenient and solvent free (Ramírez, Cuadras, Rovira, Borrull and Marcé, 2010; Kim, Moon, Hosaka, Watanabe, Teramae and Myung, 2016). Application of TD-GC-MS for quantitative and qualitative analysis of organic compounds has proved to them to good analytical procedures (Wang et al., 2015; Yang, Hsu, Chen, Young, Huang and Ku, 2017).

CHAPTER THREE: METHODOLOGY

3.1 Study design

A mixed research design was employed for this study. This involved a quantitative survey to collect data about the prevalence of various microbial populations in the different samples, as well as an experiment to determine the technological properties of selected LAB isolates in the model milk medium.

3.2 Sample origin and preparation

A total of ten independent samples (n=10) from the Gouda cheese production line were randomly collected at Sanatos Dairies (U) Limited according to the production process of Gouda cheese i.e., two samples from each of the following stages of production; (1) raw cheese milk (500 ml), (2) pasteurized cheese milk, (3) non-spiced Gouda cheese, 1 week old (500 g), (4) non-spiced Gouda cheese, 1 month old (500 g), and (5) spiced Gouda cheese, 1 month old (500 g).

All samples were collected on the same day and transferred aseptically to Makerere University Food Science microbiology laboratory and analyzed within 8 h of collection. For each sample, 25 g was weighed in a sterile bag and homogenized (230 rpm, 2 min) with 225 ml of buffered peptone water to attain 10^{-1} dilution. Further dilutions up to to 10^{-7} were prepared in peptone water using the same approach.

3.3 Microbial enumeration

An aliquot of 0.1 ml of each of the diluted samples was inoculated on the various media by spread plating, in triplicates. All plates were incubated under appropriate conditions of temperature and aeration (Table 3). After incubation, plates with 30-300 colonies were enumerated (Stuart Scientific, England) to establish microbial counts from the different samples using the following equation 1 and expressed as log cfu/g.

$$Colony\ forming\ units\ cfu/g \quad = Count \times \frac{1}{Dilution} \times \frac{1}{Sample\ volume} \qquad \text{Equation 1}$$

Table 3. Incubation conditions for various inoculated culture media

Medium	Temperature (°C)	Time	Environment	Target
Nutrient agar	30	48 h	Aerobic	Total viable counts
M17 agar	30	48 h	Aerobic	Lactococci
Baird Parker agar	37	24 h	Aerobic	Staphylococci
VRBL agar	30	24 h	Aerobic	Coliforms/ *E. coli*
MRS agar	30	48 h	Anaerobic	Total LAB
Potato dextrose agar	25	3-5 days	Aerobic	Yeasts & molds

3.4 Microbial isolation, purification and presumptive identification

After enumeration of viable counts (section 3.2), plates containing 30-300 colonies were used for harvesting of microbial isolates which were used for further identification and characterization. The colonies with different morphologies (color, size, texture, etc.) were purified by streaking twice on the respective selective media agar and appropriately incubated as in Table 3. One colony from each of the purified cultures was inoculated in 10 ml of respective selective media broths and appropriately incubated. A 1 ml culture containing 20% sterile glycerol was stored in a sterile 1.5 ml Eppendorf tube at -25°C until further examination for cell morphologies, Gram reaction and biochemical characteristics using conventional microbiology methods for presumptive identification.

3.4.1 Gram staining

Gram staining, catalase and oxidase tests were performed according to the procedure of Mugampoza et al. (2020). With the help of a dropper, one drop of peptone water was transferred on a slide into which a microbial colony was dispersed to produce a smear. The smear on the slide was allowed to dry at ambient temperature (10 min) and passed through a Bunsen flame 3-5 times (heat fix). It was stained for 1 min with crystal violet. Crystal violet was rinsed off and Lugol's iodine added (30 s). Iodine was washed off using tap water and then 96% ethanol was applied for 1 min and washed off the excess, with tap water. The smear was eventually stained for 30 s with safranin. Excess safranin was rinsed off with tap water and the slide was air-dried at ambient temperature for 10 min. A light microscope at a magnification of X1000 was used to examine the

Gram reaction, and cell shapes and arrangement for the different microbial isolates. *E. coli* was used as the Gram-negative control while *S. aureus* was the Gram-positive control.

3.4.2 Catalase test

An isolated single colony was picked from the plate using a wire loop and transferred onto a sterile slide. Then, H_2O_2 (30%, v/v) was applied onto the culture on the glass slide and observed for any bubbles. Appearance of gas bubbles indicated that the isolate was catalase positive while absence of bubbles indicated a negative catalase test. *Pseudomonas flourescens* and *Lactobacillus plantarum* obtained from the Department of Food Science, Nutrition and Biotechnology, Makerere University. The cultures included as the catalase positive and negative reactions, respectively.

3.4.3 Oxidase test

A strip was used to carry out the oxidase test. One end of the strip paper was rolled on colonies from a plate grown for 24 h and then left to stand for 1 min. A positive oxidase test was confirmed by observation of a color change of the strip from pink to purplish-black. The control samples included *Pseudomonas flourescens* and *Lactobacillus plantarum* for confirmation of oxidase positive and negative controls, respectively.

3.5 Confirmation of bacterial species using molecular methods

For molecular identification of the bacterial species, 16S rDNA sequence analysis was undertaken. Species identification was done using the basic local alignment search tool (BLAST) programme, while cluster analysis was performed to establish genetic diversity of isolates of the same species.

3.5.1 Extraction of genomic DNA

Genomic DNA was extracted using cetyl trimethyl ammonium bromide (CTAB) protocol (Mugampoza et al., 2020). Bacterial isolates were cultured in nutrient broth (24 h, 30-37^{o}C). Cells from culture broth (1 ml) were centrifuged (8000 rpm, 5 min) to recover the pellet, and washed the pellet twice (8000 rpm, 4^{o}C) in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). Then, 0.03 ml of sodium dodecyl sulphate (20%,SDS) was added followed by 0.1 ml of CTAB solution and incubated (10 min, 65^{o}C). Then, 967 µl chloroform: isoamyl alcohol (24:1) was added. The resultant solution was mixed thoroughly and spinned for 5 min at 13,000 rpm. The

upper phase (0.5 ml) was put in a clean micro centrifuge tube, 0.5 ml of cold isopropanol added and mixed (1 min) to precipitate DNA. DNA was recovered by centrifugation (4^{o}C, 5 min, 13,000 rpm). The DNA was washed twice in 0.5 ml of absolute ethanol, dried at ambient temperature for 30 min, re-suspended in 0.1 ml TE buffer and kept at $^{-}$25^{o}C until analysis.

3.5.2 PCR amplification of 16S rRNA

V3F and V3R primers were used to amplify the V3 region of 16S rRNA to give a PCR amplicon of 200 bp. Reaction mix of 50 µl was constituted from 5 µl of PCR buffer (10X); 2.5 mM deoxynucleotide triphosphates; 0.2 pmol/µl forward and reverse primers; 1.25 U of *Taq* DNA polymerase; and 1 µl of DNA. The tube was inserted in a thermocylcer and amplified as follows: DNA denaturation (94^{o}C, 5 min), initial annealing temperature (66°C), this was decreased by 1°C every cycle for 10 cycles. Finally, 20 cycles were conducted at 56°C. Extension for each cycle was done (3 min, 72°C). Final extension was done (10 min, 72°C).

3.5.3 Gel Electrophoresis

PCR amplicon (10 µl) was mixed with a loading dye (6X, 2 µl) and transferred on a 2% agarose gel containing ethidium bromide (0.2 µg/ml). Electrophoresis was done in 1X TAE buffer at 75 V, 2 h. A 100 bp DNA marker was used as the standard. The gel was visualized using a UV trans illuminator, and bands recorded with Quantity One Gel Doc software (BioRad).

3.5.4 Sequencing and database search

Purification and sequencing of PCR amplicons was done at Macrogen Meibergdreef 31, Amsterdam (Netherlands). The nucleotide sequences obtained were used to establish the closest known relatives of the isolates using the BLAST search tool and the NCBI gene bank.

3.6 Determination of antimicrobial activity

3.6.1 Agar overlay method

Antimicrobial activity of the LAB isolated from different stages of Gouda cheese production was conducted against selected indicator bacterial strains (Table 4) for preliminary screening of isolates with potential activity. For each sample, 10-fold dilutions of the culture grown at 30^{o}C for 24 h on MRS media was performed to obtain about 50 colonies on a Petri dish. Colonies were overlaid using 0.5% nutrient agar, spiked with 10^5 cfu/ml of the test bacterium. The medium was left to

solidify at ambient temperature (10 min), incubated further (37ºC, 24 h), and then examined for colonies surrounded by a halo.

Table 4. Bacterial species used for antimicrobial activity screening of LAB isolated from Gouda

Bacterial species	Obtained from
Escherichia coli	Makerere University, Department of Food Science & Nutrition
Salmonella enterica	"
Staphylococcus aureus	"

3.6.2 Antimicrobial activity by spotting method

The protocol of Mugampoza et al. (2020) was followed to demonstrate antimicrobial activity of the Gouda LAB isolates using cell-free supernatant (CFS) assays. Each of the LAB isolates was cultured in 10 ml MRS broth (30ºC, 24 h). The culture was spinned for 5 min at 8000 rpm to obtain the cell-free supernatant (CFS). The CFS was sterilized by filtration through a 0.45 μm membrane prior to use in the experiment.

The indicator bacterial strains (Table 4) were cultured in nutrient broth (10 ml) at 37ºC for 24 h and diluted up to 10^{-5}. Then, 0.1 ml spread on nutrient agar using an L-shaped disposable spreader. Inoculated plates were spotted with 20 μl of CFS and dried at ambient temperature for approx. 10 min. Sterile MRS broth was used as the negative control. All plates were incubated (37ºC, 24 h) and assessed for halos around the spot.

3.7 Growth, acid and aroma production capacity of the dominant LAB isolated from Gouda cheese

3.7.1 Design of the milk model experiment

Full cream UHT cow's milk (Jesa) was used to determine the growth, acid and aroma production properties of the selected lactic acid bacterial species according the protocol of Martín-Platero et al. (2008). Milk sample (80 ml) was pipetted in a 250 ml conical flask; subjected to the following treatments (final concentration, 3 log CFU/ml); (1) single Gouda LAB isolates, (2) mixed Gouda

LAB isolates belonging to different species. All flasks were incubated at 30^{o}C, 48 h and kept for 1-4 weeks to simulate Gouda cheese ripening conditions. UHT without microbial inoculation was used as the negative control. Sample aliquots were collected at various time intervals every six hours from 0 hour to 48 hours in 10 ml universal tubes and enumerated for viable counts (growth) as described in section 3.2, and for measurement of pH & percent titratable acidity and aroma analysis.

3.7.2 pH determination

A Hanna pH meter (Hanna Instruments, Singapore) was used to measure pH. The probe was first calibrated using buffers of pH 7 and 4. Sample pH was detected by inserting the probe for 2 min into the sample to obtain a stable reading.

3.7.3 Determination of percent titratable acidity

The sample (10 ml) was titrated against 0.1 M NaOH solution. Phenolphthalein was used as the indicator

% TTA = titre volume x 10

3.7.4 Aroma profile analysis

3.7.4.1 Sample extraction

Aroma volatiles were extracted using solid phase extraction (SPE) clean-up. The sample was diluted with HPLC-grade water and filtered through Whatman No. 42 filter paper. The Oasis HLB (3 cc) cartridge was conditioned using methanol (3 ml) and equilibrated with equal volume of HPLC-grade water. The filtered sample (100 ml) was loaded onto the cartridge mounted on an Agilent SPE manifold. Thereafter, the sample was washed with 1 ml water: methanol (95:5), the cartridge was dried at ambient temperature (10 min) and eluted into a sterile vial using 3 ml methanol. The sample was concentrated and reconstituted in methanol (1 ml), filtered into a 1.5 ml Agilent vial using a 0.22 μm membrane and taken to GC-MS for analysis.

3.7.4.2 Sample preparation

A Shimadzu GC-MS QP2010 Series (Japan) was used to analyze the volatile compounds in the eluate. A BPX5 GC column with 0.25 mm thick, 30 m length and 0.25 mm internal diameter was used. Injection port temperature was set at 250^{o}C, column oven was set at 60^{o}C, and the

temperature programme used to achieve a clear/precise separation was set to start at 60^{o}C held for 1 min, then ramped at 15^{o}C/minute to 300^{o}C, and held for 10 minutes. The mass spectrometer was set in a scan mode to scan fragments from M/Z 35-500 Da. The solvent delay time was set at 0-4 min, ion source temperature at 200^{o}C. NIST library was used to predict the identities of the compounds.

3.8 Statistical analysis

Means and standard deviations were analyzed for variance (ANOVA) using SPSS. Means were separated by the LSD test using XLSTAT at $p<0.05$. Correlation analysis between aroma profiles of the LAB cultures in the milk model versus single or mixed culture treatments were performed using principal component analysis (PCA).

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Introduction

This work aimed to determine the diversity of microflora at different stages of Gouda cheese production, in order to evaluate the dominant organisms that enter the cheese production process, and to study their prospective contributions to the product quality characteristics. Conventional microbiological protocols were employed to enumerate and for presumptive identification of the different microbial genera. Species identification was attained using 16S rDNA sequence analysis and genetic diversity of the dominant bacterial established using UPGMA clustering. Antimicrobial activity of dominant LAB isolated from Gouda was established by agar overlay and disc diffusion assays, whereas growth, acid and aroma production capacities of the dominant LAB isolates was assessed *in vitro* in UHT whole milk as described in section 3.6.

4.2 Diversity of microorganisms that occur at different stages of Gouda cheese production

4.2.1 Viable microbial counts

Overall, raw milk had the highest level of the microbial parameters whereas pasteurized milk had the lowest (Table 5). Bacteria, yeasts and molds were enumerated in all the samples except pasteurized milk, which only contained bacteria. The various microbial parameters were significantly different at different stages of Gouda cheese production process ($p<0.05$). Total LAB and *Streptococci/ Lactococci* were highest in raw milk (7.53-7.77 log CFU/ml) and lowest in non-spiced Gouda cheese matured for one week (2.34-2.37 CFU/g) (Table 5). Total coliforms, *E. coli* and *Staphylococcus* spp. were highest in raw milk and non-spiced Gouda cheese matured for one week, and lowest in pasteurized milk (Table 5). Also, the results in Table 5 indicated that yeasts and molds were only detected in raw milk, and in spiced and non-spiced Gouda cheese matured for one month (2.31-4.96 CFU/ml).

Table 5. Microbial counts (log CFU/g) of raw and pasteurized milk, and Gouda cheese obtained from Sanatos in 2021

Sample	TPC	Total LAB	*Streptococci/ Lactococci*	Total coliforms	*E. coli*	*Staphylococcus*	Yeast and molds
Raw Milk	7.79 ± 0.03^{a}	7.77 ± 0.01^{a}	7.53 ± 0.03^{a}	7.38 ± 0.05^{a}	4.02 ± 0.02^{a}	6.19 ± 0.06^{b}	4.96 ± 0.02^{a}
Pasteurized Milk	2.59 ± 0.02^{c}	2.37 ± 0.09^{d}	2.34 ± 0.07^{b}	1.52 ± 0.00^{d}	0.31 ± 0.00^{c}	2.45 ± 0.02^{d}	0.00 ± 0.00^{c}
Gouda (1 week)	7.43 ± 0.01^{b}	6.19 ± 0.02^{c}	7.39 ± 0.03^{a}	1.60 ± 0.13^{d}	0.00 ± 0.13^{d}	6.49 ± 0.01^{ab}	0.00 ± 0.00^{c}
Spiced Gouda (1 month)	7.71 ± 0.16^{ab}	7.02 ± 0.09^{b}	7.66 ± 0.15^{a}	2.17 ± 0.06^{c}	2.17 ± 0.00^{b}	6.94 ± 0.21^{a}	2.39 ± 0.13^{b}
Gouda (1 month)	7.83 ± 0.05^{a}	7.12 ± 0.01^{b}	7.42 ± 0.04^{a}	5.26 ± 0.01^{b}	0.00 ± 0.00^{d}	5.29 ± 0.06^{c}	2.31 ± 0.00^{b}

Values are means of triplicate determinations. Values in columns with different superscript letters are significantly different (p>0.05).

The high total plate counts in raw milk could be explained by the inadequate pasteurization of the milk, dirty milking equipment, soiled udders, and/or milking of mastitic animals (Swai and Schoonman, 2011). The data were in agreement with those of Aljewicz et al. (2016) who suggested that total viable counts and non-starter lactic acid bacterial counts during ripening of Gouda cheeses range from 3.85 to 10.10 log CFU/g, and 8.35 to 9.22 log CFU/g, respectively. While analyzing the microbiological quality of processed cheese in Khartoum, Sudan, Suleiman et al. (2011) also reported 1.79 to 2.40 log CFU/g of coliforms while yeasts and molds ranged from 0.17 to 1.43 log CFU/g. Amran and Abbas (2011) reported the levels of *Staphylococcus* spp., coliforms, yeasts and molds in local Yemeni cheese to be 11.8, 13.4, 11.8 and 8.9 log CFU/g, respectively.

The low microbial load detected in pasteurized milk in the current work may be due to thermal destruction of microorganisms initially present in the milk (Wangalwa et al., 2016). In contrast, the higher microbial load in raw milk and non-spiced Gouda cheese matured for one week could be explained by unhygienic handling of milk during milking and possible manual handling of the freshly processed cheese whose composition is still favorable for microbial proliferation (Hayes and Boor, 2001; Mufandaedza et al., 2006).

In addition, the presence of coliforms in all tested samples could be attributed to cross-contamination, poor hygienic handling of milk and/or unhygienic manual milking process. Whereas *E. coli* is a major coliform usually found in raw milk (Wanjala et al., 2018), the low *E. coli* count obtained from this study relative to the total coliform counts implies presence of other coliform bacteria is the tested samples. Studies reported other coliforms such as *Salmonella* spp. and *Listeria* spp. in milk and dairy products, which could partly explain the results from this study (Aygun and Pehlivanlar, 2006; Hadrya et al., 2012; Hosseini et al., 2014 ; Keba et al., 2020).

Lactic acid bacteria are native to milk and dairy products. During fermentation, they produce flavor compounds as secondary metabolites (Zacharof and Lovitt, 2012; Mukisa and Kiwanuka, 2017). They also produce antimicrobial compounds such as lactic acid which have a preservative role on fermented dairy products through activity against spoilage and pathogenic microorganisms (Schlegelova et al., 2003; Azhari, 2010; Zacharof and Lovitt, 2012). Therefore, presence of high

counts of total LAB and streptococci/lactococci in all the tested samples could be a desirable attribute. Given that the number of these organisms was lowest in pasteurized milk implies high efficacy of the pasteurization process, with the exception of thermoduric LAB which could be responsible for the LAB counts detected (Table 5).

Absence of yeasts and molds in pasteurized milk could be explained by their thermal destruction during pasteurization. The commonest fungi in dairy products that contribute to fermentation and maturation of these products include *Saccharomyces cerevisiae, Candida silvae, Trichosporon asahii, Kluyveromyces marxianus* among others (Narvhus and Gadaga, 2003; Ongol and Asano, 2009). In light of the above results, there was a need to determine the species that dominate in the different microbial groups enumerated from the samples.

4.2.2 Presumptive identification of bacteria isolated at different stages of Gouda production

Overall, 125 microorganisms were isolated by streak plating on respective selective media agars as described in section 3.3 of which 121 isolates (96.8%) were bacteria and the rest were yeasts and molds. Given that the isolates were dominated by bacteria, these were studied further and results of presumptive identification and confirmation of species identification are presented in sections 4.2.2 and 4.2.3.

Pure colonies of each of the bacterial isolates were studied for colony and cell morphologies (after gram staining), catalase and oxidase reactions and classified into six (06) groups (Table 6). Isolates in each of the groups were presumptively identified according to the scheme given at www.bacteriainphotos.com.

Majority of the isolates were pathogenic bacteria presumptively identified as *Staphylococcus* spp. (26.5%), *Streptococcus,* or *Enterococcus* (23.9%), *Klebsiella, Enterobacter, Eschericia coli, Salmonella, Proteus, Serratia,* or *Yersinia* (19%) and *Bacillus, Streptomyces, Listeria* or *Corynebacterium* (9.9%). Presumptive *Lactobacillus* or *Clostridium* were 14.1% (Table 6).

Table 6. Presumptive identification of bacteria isolated from milk and Gouda cheese obtained from Sanatos in 2021

Group	Catalase	Oxidase	Gram reaction	Cell shape	Number (%) of isolates	Presumptive identification
1	+	-	+	cocci	32 (26.5)	*Staphylococcus*
2	-	-	+	cocci	29 (23.9)	*Streptococcus, Enterococcus*
3	+	-	-	rods	23 (19.0)	*Yersinia, E. coli, Enterobacter, Salmonella, Klebsiella, Proteus, Serratia,*
4	-	-	+	rods	17 (14.1)	*Lactobacillus, Clostridium*
5	+	-	+	rod	12 (9.9)	*Listeria, Corynebacterium, Bacillus, Streptomyces,*
6	+	-	+	rods	8 (6.6)	Not identified
				Total	**121**	

Pathogenic bacteria could have entered the Gouda cheese production process as a result of cross contamination by either cross contamination from the processors or cow dung during milking, since milk is the principal ingredient in cheese making (Papademas, and Robinson, 1998). Majority of the presumptively identified pathogenic bacteria have detrimental effects on cheese quality and safety properties, unless acted upon by the antimicrobial agents therein as a result of growth of fermentative bacteria including lactic acid bacteria (Durango-Zuleta et al., 2022). The prevalence of pathogenic bacteria could also be accounted for by their thermal resistance especially for the thermoduric coliforms such as *E. coli* (Machado et al., 2017). These organisms also produce antimicrobial agents, which enable them to compete with other microorganisms during dairy fermentations (Gálvez et al., 1986; Manzano et al., 1993). Part of the study results were not descriptive for the genera identified within majority of the groups, except for group 1 (*Staphylococcus* spp.), therefore, 16S rDNA sequence analysis (section 4.2.3) was performed to

obtain reliable identification of the genus in each group and to establish the different species in each of the identified genera.

4.2.3 16S rDNA sequence analysis for species identification of bacteria isolated from Gouda cheese

Out of the 121 bacterial isolates presumptive identified in section 4.2.2, only 80 isolates were resuscitated from -20ºC freezer and were able to grow and examined in this section. PCR results for amplification of the gene are shown in Fig. 2.

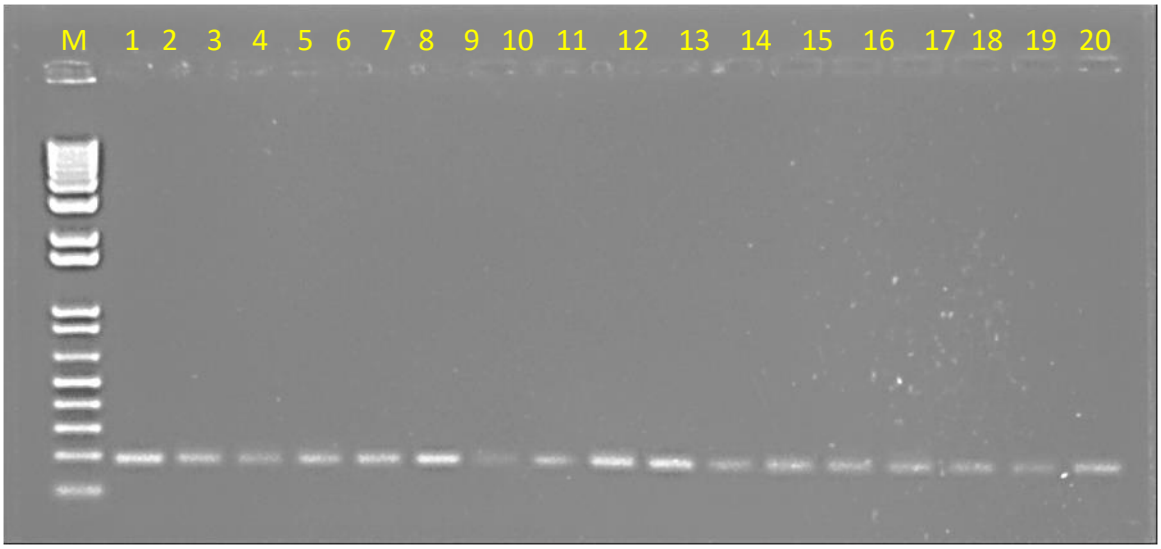

Figure 2**.** Representative gel image showing PCR amplification of 16S rDNA of bacteria isolated from Gouda cheese. Amplicons were run on 2% agarose gel in 1X TAE buffer (75 V, 2 h)

M – 100 bp molecular marker
1 to 5, isolates from raw milk

6-10, isolates from pasteurized milk

11-15, isolates from non-spiced Gouda cheese matured for 1 month

16-20, isolates from spiced Gouda cheese matured for 1 month

It is evident from Figure 2 that a 200 bp expected PCR product was obtained for all the 80 tested isolates implying good accuracy of the protocol. After PCR, a 10 µl aliquot of each sample containing the PCR product was sent to Macrogen Meibergdreef (Netherlands) for purification

and sequencing. The sequence results were subjected to the BLAST programme to retrieve the closest known relatives of the isolates in the NCBI database (Table 7).

Table 7. Results of BLAST sequences obtained from PCR amplification of 16S rDNA of bacteria isolated from Gouda cheese obtained from Sanatos in 2021

Group	Gene accession	Close relative of	Number of isolates	Proportion (%)	E-value	%ID
1	CP072327.1	*Klebsiella aerogenes*	4	5.0	3e-26	93.4
2	FJ626282.1	*Hafnia paralvei*	6	7.5	4e-69	96.9
3	MT324263.1	*Salmonella bongori*	2	2.5	2e-67	96.4
4	MN725738.1	*Klebsiella michiganensis*	1	1.3	1e-43	87.7
5	KX156182.1	*Klebsiella oxytoca*	1	1.3	1e-68	97.5
6	MT338545.1	*Enterobacter cloacae*	2	2.5	3e-75	100
7	KX759088.1	*Klebsiella pneumoniae*	10	12.5	2e-67	96.9
8	KJ806411.1	*Shigella sonei*	1	1.3	5e-31	93.4
9	MT338545.1	*Enterobacter cloacae*	2	2.5	1e-67	96.4
10	LC383590.1	*Burkholderia sp.*	1	1.3	0.001	91.3
11	MK294289.1	*Enterobacter hormaechei*	1	1.3	2e-49	94.9
12	CP003416.1	*Salmonella enterica*	2	2.5	2e-27	92.6
13	HQ333013.1	*Bacillus cereus*	6	7.5	5e-71	97.6
14	JQ511429.1	*Staphylococcus succinus*	2	2.5	7e-16	90.8

15	GQ169122.1	*Staphylococcus equorum*	1	1.3	2e-67	96.9
16	KY615348.1	*Bacillus pseudomycoides*	2	2.5	1e-73	99.4
17	KY002647.1	*Bacillus anthracis*	2	2.5	3e-68	96.9
18	CP092906.1	*Aeromonas hydrophila*	1	1.3	1e-69	98.1
19	OK355389.1	*Staphylococcus epidermidis*	1	1.3	5e-13	94.6
20	MG726042.1	*Bacillus wiedmannii*	1	1.3	1e-37	90.8
21	ON090364.1	*Lactococcus garvieae*	1	1.3	3e-73	98.8
22	MT573003.1	*Leuconostoc citreum*	6	7.5	5e-57	94.2
23	KT626400.1	*Streptococcus lutetiensis*	7	8.8	5e-76	99.4
24	MT473506.1	*Enterococcus italicus*	1	1.3	4e-68	98.7
25	MH666046.1	*Lactococcus lactis*	10	12.5	2e-72	100
26	MT231812.1	*Lactobacillus plantarum*	4	5.0	4e-58	94.3
27	MN703687.1	*Pseudomonas fragi*	1	1.3	3e-71	98.7
28	MT525278.1	*Bacillus subtilis*	1	1.3	5e-58	94.2
		Total	**80**			

The BLAST results revealed identifiable sequences for all the 80 isolates with percent identities ranging from 90.8 to 100% (Appendix I). Eleven isolates (13.8%) gave percent identities lower than the minimum value of 95% implying possible mismatch. In spite of this, there were matching sequences for all the 80 isolates examined which shows good reproducibility of PCR. A total of 28 bacterial species were delineated by the BLAST programme. The dominant species (12.5%) were found to be *Klebsiella pneumonia* and *Lactococcus lactis* (Table 7). These species were recorded from all tested samples.

The major concern was detection of various pathogenic bacteria including *Salmonella, Shigella, Bacillus, Staphylococcus, Klebsiella* and *Aeromonas* spp. Presence of coliform bacteria such as *Enterobacter* and *Enterococcus* spp. is indicative of fecal contamination, as they normally inhabit the gut. Raw milk and water have been reported as the major sources of *Salmonella, Shigella, Aeromonas* and *S. aureus* in cheese (Pasquali et al., 2022). *S. aureus* could also be introduced from handlers since it is part of the common skin microbiota. Pathogenic species of *Klebsiella* are often linked to nosocomial infections due to their multi-drug resistance properties (Gelbíčová et al., 2021). Presence of these organisms in cheese is the result of dirty udders and bovine mastitis in cows (Massé et al., 2020).

Lactococcus lactis, *Leuconostoc* spp., *Streptococcus* spp. and *Lactobacillus plantarum* were detected in 1-4 weeks matured cheese samples. These organisms are known to impart desirable quality characteristics in fermented foods (Minervini et al., 2017; Kıvanç, and Yapıcı, 2015; Todorov, and Franco, 2010).

Data from 16S rDNA sequence analysis generally agreed with those attained by conventional microbiology methods (Section 4.2.2). For instance, *Klebsiella* spp. was found to be among the dominant organisms in the viable counts study (section 4.2.2) which is similar with the BLAST data. In fact, all the bacterial genera which were presumptively identified in section 4.2.2 were also confirmed by PCR, except for *E. coli*, *Proteus*, *Serratia*, *Yersinia*, *Listeria*, *Corynebacterium* and *Clostridium* which were only presumptively identified by conventional microbiological methods. These however, could have been among the samples that could not be resuscitated. As PCR is regarded to be a precise and reliable tool for microbiological identification (Kuypers and Jerome, 2017), the latter bacteria were therefore ruled out to be present in the samples examined.

However, given that fecal coliforms were detected by PCR (Table 7), implies that some of the latter organisms such as *Escherichia coli*, which belong to the coliform group, could have also been present but not isolated in the pool of organisms examined. Based on these results, it was evident that the bacterial flora at different stages of Gouda cheese production was diverse and comprised of desirable and undesirable organisms. Thus, desirable LAB isolates including *Lactobacillus, Lactococcus* and *Streptococcus* were examined further for genetic diversity to establish whether or not there were different strains of these organisms isolated at different stages of the cheese production process, which could lead to differences in their phenotypic expression including starter culture, aroma and antimicrobial properties. Variations in the latter properties could lead to variations in product quality attributes amongst different batches thereby leading to differences in customer perceptions of the cheese.

4.2.4 UPGMA cluster analysis

UPGMA clustering was conducted on the 10 *Lactococcus lactis*, 08 *Streptococcus lutetiensis* and 03 *Lactobacillus plantarum* isolates. These three bacterial species were only detected in non-spiced Gouda cheese ripened for one week and spiced Gouda cheese ripened for one month. The dendrogram (Fig. 3) delineated the isolates according to the three different genera showing that the latter organisms have diverse genetic composition.

Lc. lactis isolates from non-spiced Gouda cheese matured for one week (Gw 1, 2, 3, 4, 5, 6 and 7) were clustered together with *Lc. lactis* isolates from spiced Gouda cheese matured for one month (GSm 12 and 13, Fig. 3) implying close genetic relatedness. All *Strep. lutetiensis* isolates (GSm 1-8) were only obtained from spiced Gouda cheese matured for one month (GSm). Similarly, all *Lb. plantarum* isolates (GSm 9, 10 and 11) were only obtained from spiced Gouda cheese matured for one month (GSm). These findings propose that the survival of *Strep. lutetiensis* and *Lb. plantarum* in matured cheese is enhanced by the presence of spices (Fig. 3). None of these LAB organisms was detected in raw cheese milk, in spite of high LAB counts obtained by enumeration on MRS and M17 agar (Table 5). In addition, pasteurized milk which had the lowest counts of total LAB/streptococci/lactococci (Table 5) also revealed the lowest LAB species by PCR, which is congruent between these two parts of the study. In fact, only *Lc. lactis* (PM-1) was detected in pasteurized milk (Fig. 3). This demonstrates that pasteurization is efficient at reducing levels of

secondary LAB flora. This is desirable because non-starter LAB retard the activity of desirable LAB starter cultures.

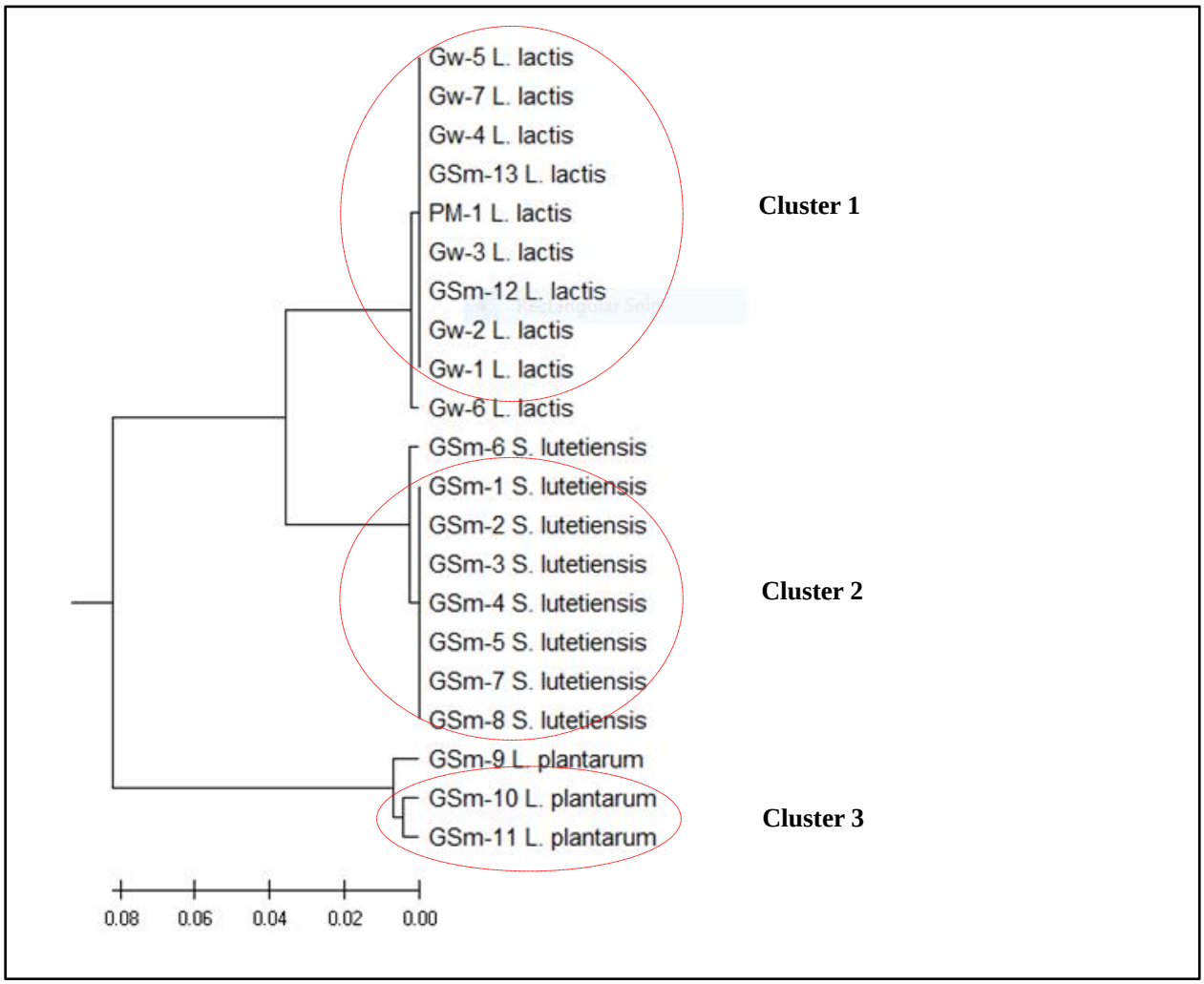

Figure 3. Dendrogram showing UPGMA clustering of *Streptococcus lutetiensis*, *Lactococcus lactis* and *Lactobacillus plantarum* isolated from Gouda cheese. Bacteria were isolated from: PM (pasteurized milk), Gw (non-spiced Gouda cheese matured for 1 week), GSm (spiced Gouda cheese matured for one month).

Lactococcus lactis and *Lactobacillus plantarum* are known to impart desirable properties in fermented foods (Gustaw et al., 2021; Liu et al., 2004). All of the 10 *Lc. lactis* and 03 *Lb. plantarum* isolates highlighted in Figure 3 were therefore probed further for antimicrobial activity against selected pathogenic bacteria, growth, acid and aroma production properties in a UHT milk

model in order to evaluate their prospects for application as bio-preservatives and/or starter cultures/culture adjuncts. The results are presented in sections 4.3-4.4.

4.3 Antimicrobial activity of *L. plantarum* and *L. lactis* isolated from Gouda cheese

Indicator bacteria including *Escherichia coli*, *Staphylococcus aureus* and *Salmonella enterica* were used to screen for antimicrobial activity of the 10 *Lactobacillus plantarum* and 03 *Lactococcus lactis* isolated from Gouda cheese, using spotting and agar overlay assays.

4.3.1 Agar overlay assay

Each of the Gouda LAB isolates formed a zone of clearing (halo) on the agar overlay Petri dishes (Fig. 4) implying that they all had activity against indicator bacteria. However, the level of reaction as demonstrated by the size of the clear halo varied with the Gouda LAB isolate and indicator bacteria tested (Table 8) highlighting possible differences in the type or quantity of antimicrobial agent(s), which could be produced by the tested Gouda LAB isolates.

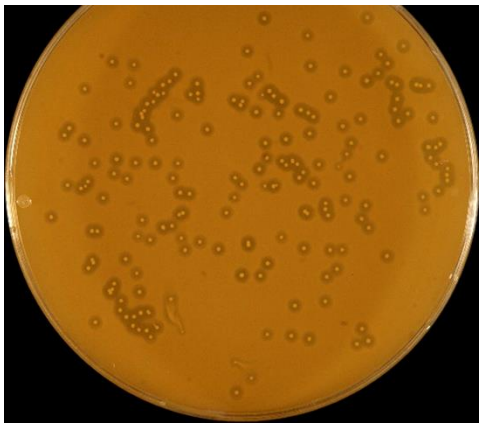

Figure 4. Antimicrobial activity of *Lactococcus lactis* Gw-5 obtained from non-spiced Gouda cheese matured for one week, against *E. coli*. Activity is shown by a clear halo surrounding *Lc. lactis* colonies.

Table 8. Antimicrobial activity of *L. plantarum* and *L. lactis* isolated from Gouda cheese obtained from Sanatos in 2021. Results by the agar overlay assay.

S/N	Isolate	Close relative of	Antimicrobial activity against		
			Escherichia coli	*Salmonella enterica*	*Staphylococcus aureus*
1	GSm-9	*Lactobacillus plantarum*	+++	++	+
2	GSm-10	*Lactobacillus plantarum*	+++	++	+
3	GSm-11	*Lactobacillus plantarum*	+++	++	+
4	GSm-12	*Lactococcus lactis*	++	++	+
5	GSm-13	*Lactococcus lactis*	++	++	+
6	Gw-1	*Lactococcus lactis*	+++	++	+
7	Gw-2	*Lactococcus lactis*	+++	++	+
8	Gw-3	*Lactococcus lactis*	++	++	+
9	Gw-4	*Lactococcus lactis*	++	++	+
10	Gw-5	*Lactococcus lactis*	+++	++	-
11	Gw-6	*Lactococcus lactis*	++	++	-
12	Gw-7	*Lactococcus lactis*	+_	++	+
13	PM-1	*Lactococcus lactis*	+++	++	+

Slight (+), moderate (++), strong (+++) reaction, or absence (-) of a clear zone of inhibition around the colony of indicator bacteria (Figure 3)

PM = pasteurized milk
Gw: non-spiced Gouda cheese matured for one week
GSm: spiced Gouda cheese matured for one month

Overall, *E. coli* was the most sensitive while *Staph. aureus* was the least inhibited indicator bacteria. *Lb. plantarum* had the same level of inhibition for *E. coli* whereas *Lc. lactis* had variable inhibition levels against *E. coli*. For instance, *Lc. lactis* isolated from non-spiced Gouda matured for one week was generally inhibitory against *E. coli* compared to isolates obtained from spiced Gouda cheese matured for one month, which could be attributed to spice induced mutations against the antimicrobial activity of *Lc. lactis* (Table 8). *Salmonella* spp. had same level of reaction to all the tested Gouda LAB isolates. Similarly, *Staph. aureus* had same level of reaction to the tested Gouda LAB isolates except for *Lc. lactis* Gw-5 and Gw-6 which did not show activity against this indicator bacterium. These results suggest that Gram-negative bacteria were more sensitive to antimicrobial agents produced by Gouda LAB isolates than Gram-positive bacteria, most likely due to variances in their cell wall structure (Tavares et al., 2020).

4.3.2 Antimicrobial activity using the spotting assay

In order to confirm that the Gouda LAB isolates confer antimicrobial activity by producing metabolites that are inhibitory to the tested indicator bacteria, the latter isolates were developed in MRS broth which was treated as in section 3.5.2 to eliminate cells. Cell-free supernatant (CFS) was spotted on plates inoculated with the indicator bacteria and incubated at 37ºC for 24 h. Plates were assessed for a zone of clearing around the spot, which was indicative of presence of antimicrobial agents in the CFS. The outcomes of this evaluation are indicated in Fig. 5 and Table 9.

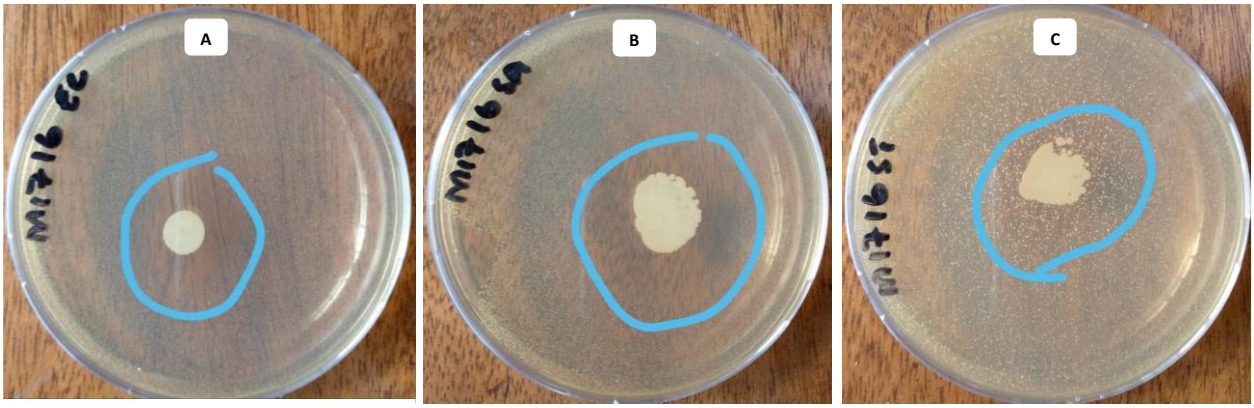

Figure 5. Antimicrobial activity of cell-free supernatant of *Lc. lactis* Gw-5 isolated from non-spiced Gouda cheese matured for one week, against (**A**) *E. coli*, (**B**) *Salmonella enterica*, (**C**) *Staphylococcus aureus*. Assessment was conducted using the spotting method. Activity in A and B is shown by presence of a clear zone of inhibition, while lack of activity (C) is shown by absence of the zone of clearing around the spot.

Table 9. Antimicrobial activity of cell-free supernatant of *Lb. plantarum* and *Lc. lactis* isolated from Gouda cheese obtained from Sanatos in 2021 against *E. coli*, *Salmonella enterica* and *S. aureus*

S/N	Isolate ID	Close relative of	Antimicrobial activity of CFS against		
			E. coli	*Salm. enterica*	*Staph. aureus*
1	GSm-9	*Lactobacillus plantarum*	-	-	-
2	GSm-10	*Lactobacillus plantarum*	-	-	-
3	GSm-11	*Lactobacillus plantarum*	-	-	-
4	GSm-12	*Lactococcus lactis*	-	-	-
5	GSm-13	*Lactococcus lactis*	-	-	-
6	Gw-1	*Lactococcus lactis*	-	-	-
7	Gw-2	*Lactococcus lactis*	-	-	-
8	Gw-3	*Lactococcus lactis*	-	-	-
9	Gw-4	*Lactococcus lactis*	-	-	-
10	Gw-5	*Lactococcus lactis*	+	+	-
11	Gw-6	*Lactococcus lactis*	-	-	-
12	Gw-7	*Lactococcus lactis*	-	-	-
13	PM-1	*Lactococcus lactis*	-	-	-

(-) shows that not any clear zone of inhibition was seen
(+) a clear area of inhibition of 25 mm against *E. coli* and 20 mm against *Salmonella* was observed

None of the CFS showed activity against *Staph. aureus* which was similar with the outcomes obtained by the agar overlay method (section 4.3.1). Surprisingly, all *Lb. plantarum* isolates which showed high level of activity against *E. coli* and *Salmonella* in the agar overlay method lost activity in the CFS spot assay. *Lc. lactis* also exhibited very low activity against *E. coli* and *Salmonella* contrary to the data obtained by agar overlay protocol. In fact, only one *Lc. lactis* isolate (Gw-5) showed activity against *E. coli* and *Salmonella* (Table 9). Reasons for these results require further investigation. However, it is possible that activity of the CFS from *Lc. lactis* Gw-5 against *Salmonella* and *E. coli* (both Gram negative) was due to organic acids because Gram negative bacteria are known to be more acid sensitive than Gram positive counterparts (Ammor *et al*., 2006).

Thus, *Lc. lactis* Gw-5 was selected for further studies involving growth, acid and aroma production capability in the UHT milk model medium (section 4.4). *Lb. plantarum* GSm-10 was included

from the *Lactobacillus* cluster (Fig. 3) in order to evaluate the impact of possible interaction between *Lc. lactis* and *Lb. plantarum* and whether or not this has an influence on the level of acid and/or aroma produced in fermented milk.

4.4 Growth, acid and aroma production properties of *L. lactis* and *L. plantarum* isolated from Gouda cheese

Growth interactions of inherent microorganisms in fermented foods influence their flavour and other quality properties (Hu et al., 2022). Therefore, this part of the study aimed to evaluate the likely growth interfaces (synergistic or antagonistic) amongst *Lc. lactis* and *Lb. plantarum* which had been found to be the prevailing LAB flora of ripened Gouda cheese produced in Uganda. These organisms are known to impart desirable properties in fermented foods (Wang et al., 2023). Because there were no major differences in the genetic relatedness (Fig. 3) of the LAB belonging to each of these species, only *Lc. lactis* Gw-5 was considered for this study because it had expressed a different phenotypic profile in the antimicrobial assays (sections 4.3.1-4.3.2). Thus, *Lc. lactis* Gw-5 was assessed for growth in model UHT milk medium as a single culture and as a co-culture with *Lb. plantarum* GSm-10. These organisms were also assessed for acid and aroma production capacities, the results of which are indicated in sections 4.4.2-4.4.3. UHT is normally used for studying microbial interactions in dairy matrices because its fermentation using inoculated LAB strains fairly simulates initial stages of cheese production (Gkatzionis *et al*., 2009). Moreover, it is a sterile medium that facilitates obtaining a homogeneous sample aliquot at different time intervals, and represents dairy matrices from which these LAB were isolated. Thus, results obtained in UHT milk would fairly reflect the behavior of these LAB isolates in other dairy-based food materials including cheese.

4.4.1 Growth kinetics

Single cultures of *Lc. lactis* Gw-5 and *Lb. plantarum* GSm-10 had similar growth kinetics with a short lag phase (0-3 h), exponential phase (4-24 h) and stationary phase (25-48 h) (Fig. 6). The growth rates of *Lc. lactis* Gw-5, *Lactobacillus plantarum* Gsm-10 and mixed culture were not significantly different ($p>0.05$) at 6, 12, 18, 24 and 48 hours as revealed by the error bars. In general, *Lc. lactis* Gw-5 as single or co-culture had faster growth rate than *Lb. plantarum* GSm-

10. For all culture treatments, maximum growth occurred at 24 h with single (8.5 log CFU/ml) and co-culture (9 log CFU/ml) of *Lc. lactis* showing the highest growth than single culture of *Lb. plantarum* GSm-10 (7.8 log CFU/ml). Overall, *Lc. lactis* Gw-5 and *Lb. plantarum* had indistinguishable growth patterns by the end of the experiment, all attaining a final cell count of 8.7 log CFU/ml (Fig. 6).

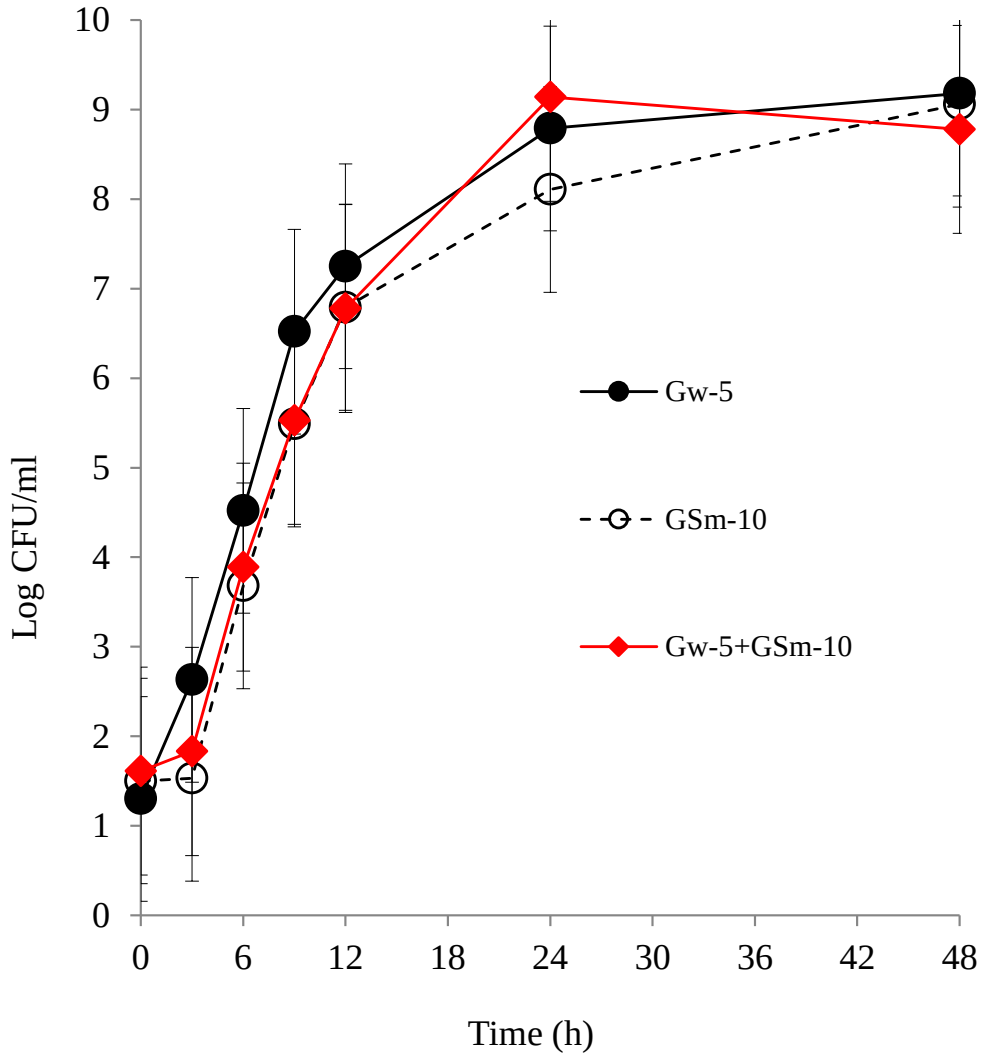

Figure 6. Changes in viable counts of single and mixed cultures of *Lactococcus lactis* and *Lactobacillus plantarum* isolated at different stages of Gouda cheese production. Single culture inoculations: *Lc. lactis* Gw-5 (solid line, black circle); *Lb. plantarum* GSm-10 (broken line, black circle). Mixed culture inoculation for *Lc. lactis* Gw-5 and *Lb. plantarum* GSm-10 (solid line, red

diamond). Isolates were developed in UHT whole milk at 30^{o}C. Enumeration was conducted on MRS agar. Plots are means of triplicate determinations and error bars are ± SD (p = 0.05).

4.4.2 Acid production capacity

Each of the *Lactococcus lactis* Gw-5 and *Lactobacillus plantarum* GSm-10 caused pH decrease ($p<0.05$) by the end of the experiment (Fig. 7). In single culture, *Lc. lactis* Gw-5 reduced pH from 6.62 to 4.77, whereas *Lb. plantarum* reduced pH from 6.67 to 5.27. Mixed culture of these organisms resulted in higher pH decrease from 6.68 to 4.51 but was not significantly different ($p>0.05$) from the single culture inoculation of *Lc lactis* Gw-5 at 48 h.

As expected, results of percent titratable acidity (TTA) followed a similar but opposite trend. Between 0-12 hours, there was no significant difference ($p>0.05$) in percent titratable acidity by *Lc. lactis* Gw-5, *Lb. plantarum* Gm-10 and mixed culture. However, between 24-48 hours, there was significant difference ($p<0.05$) in percent titratable acidity among the treatments with *Lc. lactis* Gw-5 producing the highest acidity while *Lb. plantarum* Gsm-10 had the lowest. Single culture of *Lc. lactis* Gw-5 which caused the highest pH decline also led to the highest percent TTA of 1.69 (Fig. 7) showing high acidification capacity of the latter. Similarly, single culture inoculation of *Lb. plantarum* GSm-10 which caused the lowest pH decline also led to the lowest percent TTA of 0.51 highlighting low acidification capability of this organism. Mixed culture inoculation had intermediate percent TTA of 0.77 suggesting that presence of *Lactobacillus plantarum* in mixed culture with *Lc. lactis* hinders acid production capability of the latter. Given that the isoelectric pH of milk is 4.6 (Kharb, 2021), this implies that, *Lc. lactis* Gw-5 in single or in mixed culture with *Lactobacillus plantarum* GSm-10 causes sufficient acidification of milk to form a curd, which is a desirable attribute of potential starter culture organisms.

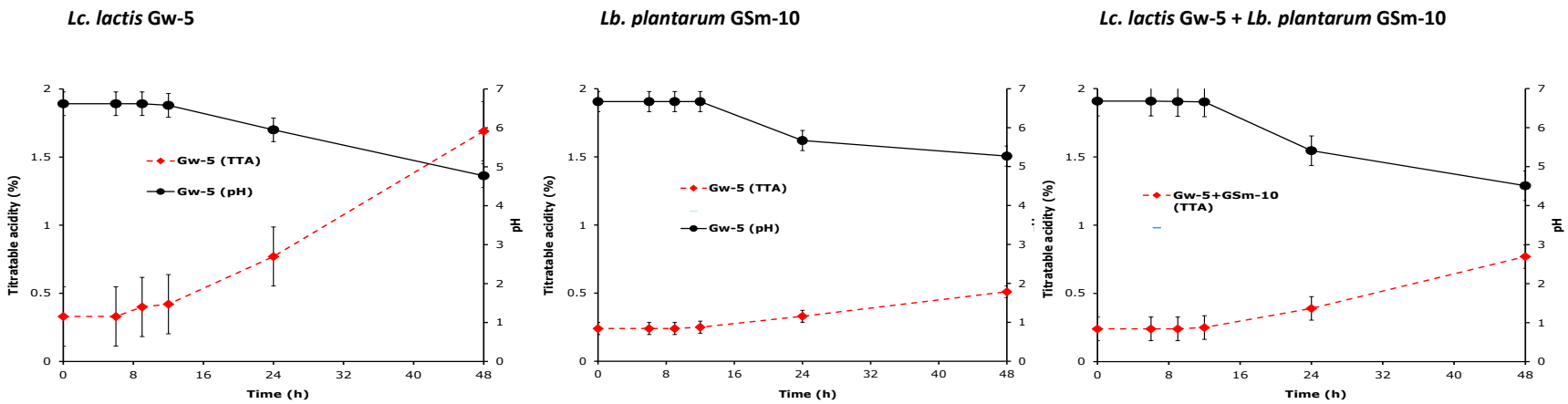

Figure 7. Changes in pH and percent titratable acidity of UHT milk spiked with *Lactococcus lactis* and *Lactobacillus plantarum* (approx. 1.5 log CFU/ml) isolated at different stages of Gouda cheese production. Isolates: *Lc. lactis* Gw-5 is *Lactococcus lactis* from non-spiced Gouda cheese matured for one week; *Lb. plantarum* GSm-10 is *Lactobacillus plantarum* from spiced Gouda cheese matured for one month. Black circle (solid line), pH; red diamond (broken line), percent titratable acidity. Plots are means of triplicate determinations and error bars are ± SD (p = 0.05).

4.4.3 Aroma production capacity

Volatile aroma compounds of UHT milk fermented by *Lactococcus lactis* Gw-5 and *Lb. plantarum* GSm-10 in single and mixed culture for 48 h, and thereafter ripened for one-week and one-month were detected using SPE GC-MS as described in 3.6.3 Un-spiked UHT milk was included as baseline control. The spectrum of compounds for UHT milk is shown in Fig. 8. Fig. 9 shows the spectrum of compounds recorded in unspiced UHT milk spiked with single and mixed culture of *Lc. lactis* Gw-5 and *Lb. plantarum* GSm-10 after one-week (A_1-A_3), and one-month (B_1-B_3) of ripening. Table 10 summarizes the major compounds detected and their respective signal intensities. The compounds were categorized as organic acids (33.3% of 33 compounds), ketones (12.1%), alcohols (12.1%), esters (36.4%) and aldehydes (6.1%) (Table 10). Overall, esters and organic acids were the major compounds detected.

In UHT milk, dodecanoic acid (3.7%), cyclohexane carboxylic acid (6%), hydroxy furanone (6.8%) and D-glucitol (19.5%) were the major compounds detected (Fig. 8, Table 10). In single cultures ripened for one-week, *Lc. lactis* Gw-5 mostly produced organic acids (benzoic acid, 27.7%, undecanoic acid, 9.9%, and octadecenoic acid, 1.1%), and esters (butyl octyl ester, 7.6%, octadecyl ester, 5.7%, 3-methylbutyl ester, 4.9%, 2-methylbutyl ester, 1.1%, and 2-ethylhexyl ester, 0.7%) (Fig. 9, Table 10). This organism also produced some alcohols (pentadecanol, 1.2% and cycloheptanol, 8.6%) and an aldehyde (tridecenal, 9.2%). On the other hand, single culture of *Lb. plantarum* GSm-10 ripened for one-week mostly produced organic acids (octanoic acid, 5.9%, benzoic acid, 6.3%, decanoic acid, 1.2%, decenoic acid, 0.3% and cyclohexane carboxylic acid, 0.09%), ketones (piperazinedione, 1.9% and pentadecandione, 0.4%), and esters (3-methylbutyl ester, 8.3%, 2-methylbutyl ester, 1%, 2,3-dihydroxypropyl ester, 1.4% and 3-pentyl ester, 0.2%) (Table 10). The organism also produced an alcohol (6-methoxy-2-hexanol, 0.4%), and an aldehyde (trihydroxybenzaldehyde, 0.8%). It was apparent that in single cultures ripened for one-week, *Lb. plantarum* GSm-10 produced more compounds (13 of 33), than *Lc. lactis* Gw-5 (11 of 33). In fact, *Lb. plantarum* GSm-10 produced more organic acids, ketones and aldehydes than *Lc. lactis* Gw-5. However, *Lc. lactis* Gw-5 produced more alcohols and esters than *Lb. plantarum* GSm-10.

In mixed cultures ripened for one week, only organic acids (benzoic acid,13.9%, octadecenoic acid, 1.4% and octadecatrienoic acid, 2%) and an ester (pentadecyl ester, 2.2%) were detected. This implies that during short-term ripening, *Lc. lactis* and *Lb. plantarum* produce a wide diversity of aroma compound in single culture and co-culture of the organisms interferes with volatile aroma production in milk.

In single cultures ripened for one-month, *Lc. lactis* Gw-5 only produced organic acids (benzoic acid, 15%, dodecanoic acid, 0.59%, and propionic acid, 4.7%) and esters (octadecyl ester, 1.1%, 2,6-dihexadecanoate, 1.6% and undecyl ester, 0.6%) (Fig. 9, Table 10). Similarly, *Lb. plantarum* GSm-10 only produced organic acids (benzoic acid, 30.6%, 9-octadecenoic acid, 3.2%, propionic acid, 6.5%) and esters (butyl octyl ester, 8.8%, 3-methylbutyl ester, 3.8%, 2,6-dihexadecanoate, 1.6%, undecyl ester, 0.6% and 2,3-propyl ester, 3.8%). It was also observed that in single cultures ripened for one-month, *Lb. plantarum* GSm-10 produced more compounds (8 of 33), than *Lc. lactis* Gw-5 (6 of 33). For instance, *Lb. plantarum* GSm-10 produced more esters than *Lc. lactis* Gw-5. However, both organisms produced the same number (03) of organic acids. Among the organic acids detected, dodecanoic acid was only found in *Lc. lactis* Gw-5 and, 9-octadecenoic acid was only detected in *Lb. plantarum* GSm-10 inoculations. Among esters, octadecyl ester was only detected in *Lc. lactis* Gw-5, whereas butyl octyl ester, 3-methylbutyl ester and 2,3-propyl ester were only detected in *Lb. plantarum* GSm-10 inoculations. This implies that in single cultures ripened for one-month, *Lb. plantarum* GSm-10 produced more compounds than *Lc. lactis* Gw-5, which was similar to results obtained at one-week ripening.

In mixed cultures ripened for one-month, only organic acids (benzoic acid, 15.7%, octadecenoic acid, 1.1% and dodecanoic acid, 1.4%), esters (4-pentadecyl ester, 2.3% and 3-pentadecyl ester, 4.5%) and the ketone 10-nonadecanone, were detected. In addition, propionic acid, 2,6-dihexadecanoate and undecyl ester were only detected in single culture inoculations of each *Lb. plantarum* GSm-10 and *Lc. lactis* Gw-5 ripened for one-month and not in their co-culture. Octadecyl ester was only observed in *Lc. lactis* Gw-5 single culture; butyl octyl ester, 3-methylbutyl ester and 2,3-propyl ester were only perceived in single culture on *Lb. plantarum* GSm-10 at one-month ripening. None of these compounds were detected in mixed culture at one-month ripening. In comparison, 10-nonadecanone, 4-pentadecyl ester and 3-pentadecyl ester were

only detected in mixed culture of *L. lactis* and *L. plantarum* and none in their single cultures at one-month ripening. These results imply that during long-term ripening a mixed culture of *Lc. lactis* and *Lb. plantarum* produce more aroma compounds (08) than during short-time ripening where only 04 compounds were detected.

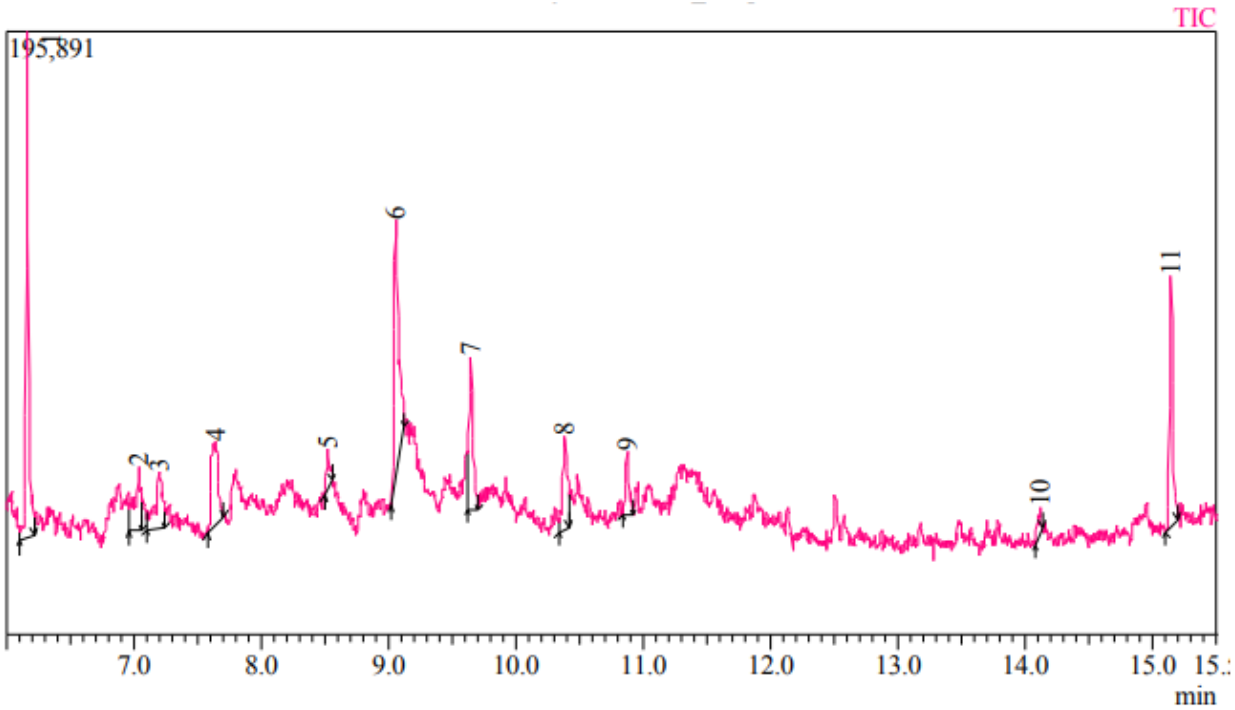

Figure 8. Spectrum of aroma ions detected in UHT milk (control) fermented for 48 h at 30^{o}C, and matured for one week at 15^{o}C to simulate Gouda cheese ripening conditions

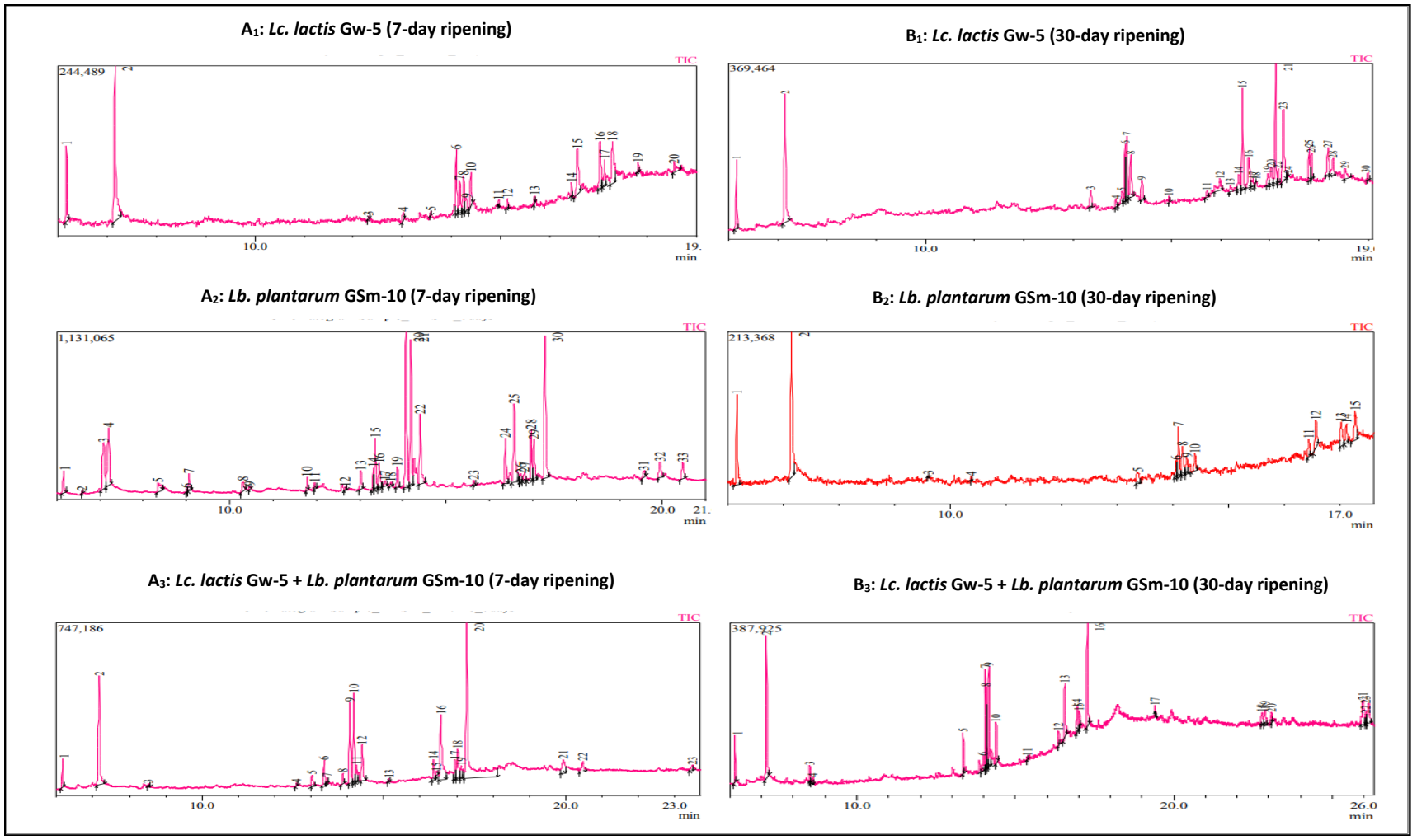

Figure 9. Aroma ions in UHT milk spiked with single and mixed culture of *L. lactis* and *L. plantarum* isolated from Gouda cheese. A_1-A_2: single culture inoculations matured for one week; A_3: mixed culture inoculation matured for one week; B_1-B_2: single culture inoculations matured for one month; B_3: mixed culture inoculation matured for one month. Isolates: *Lc. lactis* Gw-5 was isolated from non-spiced Gouda cheese matured for one week; *Lb. plantarum* GSm-10 was isolated from spiced Gouda cheese matured for one month. Inoculated samples were fermented for 48 h at 30°C and matured for 1-4 weeks at 15°C.

Table 10. SPE GC-MS percent peak intensities of the major aroma compounds in UHT milk spiked with single and mixed culture of *L. lactis* and *L. plantarum* isolated from Gouda cheese obtained from Sanatos in 2021. Isolates: *L. lactis* Gw-5 was isolated from non-spiced Gouda cheese matured for one week; *Lb. plantarum* GSm-10 was isolated from spiced Gouda cheese matured for one month. Inoculated samples were fermented for 48 h at 30^{o}C and matured for 1-4 weeks at 15^{o}C in headspace vials.

			Isolates obtained from Gouda cheese matured for one week			Isolates obtained from Gouda cheese matured for one month		
S/N	**Compound**	**UHT milk (control)**	***Lc. lactis* Gw-5**	***Lb. plantarum* GSm-10**	***Lc. lactis* Gw-5 + *Lb. plantarum* GSm-10**	***Lc. lactis* Gw-5**	***Lb. plantarum* GSm-10**	***Lc. lactis* Gw-5 + *Lb. plantarum* GSm-10**
	Organic acids							
1	Octanoic acid	ND	ND	1159706 (5.91%)	ND	ND	ND	ND
2	Benzoic acid	ND	596806 (27.69%)	1228740 (6.26%)	1483272 (13.85%)	619752 (15.02)	415005 (30.58%)	769149 (15.70%)
3	9-Octadecenoic acid	ND	23869 (1.11%)	ND	153846 (1.44%)	ND	43387 (3.20%)	55321 (1.13%)
4	Octadecatrienoic acid	ND	ND	ND	215185 (2.01%)			ND
5	Undecanoic acid	ND	214265 (9.94%)	ND	ND	ND	ND	ND
6	Decanoic acid	ND	ND	231116 (1.18%)	ND	ND	ND	ND
7	3-Decenoic acid	ND	ND	56071 (0.29%)	ND	ND	ND	ND
8	Dodecanoic acid	42351 (3.71%)	ND	ND	ND	18655 (0.45%)	ND	68497 (1.40%)
9	Propionic acid	ND	ND	ND	ND	193504 (4.69%)	88096 (6.49%)	ND
10	Cyclohexanecarboxylic acid	ND	ND	17679 (0.09)	ND	ND	ND	ND
11	Octanoic acid	68761 (6.02%)		ND	ND	ND	ND	ND
	Ketones							
12	Dihydro-4-hydroxy furanone	77338 (6.77%)	ND	ND	ND	ND	ND	ND
13	Piperazinedione	ND	ND	380702 (1.94%)	ND	ND	ND	ND
14	Pentadecandione	ND	ND	74132 (0.38%)	ND	ND	ND	ND
15	10-Nonadecanone	ND	ND	ND	ND	ND	ND	83592 (1.71%)
	Alcohols							
16	n-Pentadecanol	ND	26856 (1.25%)	ND	ND	ND	ND	ND
17	Cycloheptanol	ND	185852 (8.62%)	ND	ND	ND	ND	ND
18	6-Methoxy-2-hexanol	ND	ND	71718 (0.37%)	ND	ND	ND	ND
19	1,4-anhydro D-glucitol	222592 (19.49)	ND	ND	ND	ND	ND	ND
	Esters							
20	Butyl octyl ester	ND	162758 (7.55%)	ND	ND	ND	119706 (8.82%)	ND
21	Octadecyl ester	ND	122055 (5.66%)	ND	ND	46468 (1.13%)	ND	ND
22	3-methylbutyl ester	ND	105223 (4.88%)	1635318 (8.33%)	ND	ND	52036 (3.83%)	ND
23	2-methylbutyl ester	ND	23870 (1.11%)	202635 (1.03%)	ND	ND	ND	ND
24	2-ethylhexyl ester	ND	14789 (0.69%)	ND	ND	ND	ND	ND
25	2,6-dihexadecanoate	ND	ND	ND	ND	64764 (1.57%)	64764 (1.57%)	ND
26	Undecanoic acid (undecyl) ester	ND	ND	ND	ND	26111 (0.63%)	26111 (0.63%)	ND
27	2,3-dihydroxypropyl ester	ND	ND	280997 (1.43%)	ND	ND	ND	ND
28	3-pentyl ester	ND	ND	35187 (0.18%)	ND	ND	ND	ND
29	2,3-propyl ester	ND	ND	ND	ND	ND	51378 (3.79%)	ND
30	4-pentadecyl ester	ND	ND	ND	239065 (2.23%)	ND	ND	116939 (2.39%)
31	3-pentadecyl ester	ND	ND	ND	ND	ND	ND	221415 (4.52%)
	Aldehydes							
32	Trihydroxybenzaldehyde	ND	ND	148496 (0.76%)	ND	ND	ND	ND
33	2-Tridecenal	ND	197484 (9.16%)	ND	ND	ND	ND	ND

It was apparent that, as expected, UHT milk had fewer volatile aroma compounds compared with the fermented/ripened milk samples. In UHT milk, only dodecanoic acid, octanoic acid, dihydro-4-hydroxy furanone and 1, 4-anhydro D-glucitol were detected. With the exception of dodecanoic acid which was observed in single and mixed culture of *Lc. lactis* Gw-5 matured for one month, all the other three compounds were only detected in UHT milk.

Among the fermented/ripened samples, octanoic acid was detected in milk injected with *Lb. plantarum* GSm-10 at one-week but was undetectable at one month of ripening. Undecanoic acid and octadecatrienoic acid were detected in single and mixed cultures of *Lc. lactis* Gw-5 at one-week of ripening, respectively, but were both undetectable at one-month of ripening. This implies that beyond one week of ripening at 15^{o}C, organic acids undergo biochemical interconversion into volatile short-chain aliphatic mono-carboxylate compounds such as acetic, propionic and butyric acid (Agler et al., 2011; Yin, et al., 2019). Among the ketones, piperazinedione and pentadecandione were detected in single culture of *Lb. plantarum* GSm-10 at one-week of ripening but were undetectable at one-month. Nonadecanone was only detected in mixed culture of *Lc. lactis* Gw-5 and *Lb. plantarum* GSm-10 at one-month ripening implying possible novel synthesis from fermentation of docosahexaenoic acid (DHA) (Visali et al., 2022).

Alcohols including n-pentadecanol, cycloheptanol and 6-methoxy-2-hexanol were only detected in single and mixed cultures of *Lc. lactis* Gw-5 at one-week of ripening, none of these were detected at one-month. These results propose that for the duration of short-term fermentation of milk, *Lc. lactis* produces organic acids, ketones and alcohols but these are biochemically interconverted to esters at one-month of ripening (Paliyath et al., 2012; Forney, and Markovetz, 1971). Overall, during short-term and long-term milk fermentation, *Lc. lactis* produces more volatile aroma compounds than *Lb. plantarum*. These results agreed with the data by Helinck et al. (2004); these authors reported that *Lactococcus delbrueckii* subsp. *lactis* produces large amounts of key odorants from alcohols and/or aldehydes during amino acid catabolism, which is common in Swiss-type cheeses.

Esters were the dominant volatiles detected overall; they were mainly produced by single culture of *Lc. lactis* Gw-5 at one-week ripening followed by single culture of *Lb. plantarum* GSm-10 at one-week ripening. Of all esters produced by the latter treatments, only butyl octyl ester, octadecyl ester and 3-methylbutyl ester were subsequently detected in single and mixed culture inoculations

of *Lc. lactis* Gw-5 at one-month of ripening. This either implies degradation or biochemical interconversion of these compounds into volatile compounds such as propionic acid, butyric acid (Njokweni et al., 2021; Agler et al., 2011). In fact, new esters were produced by single cultures of *Lc. lactis* Gw-5 and *Lb. plantarum* GSm-10 at one-month ripening. This was the case for 2,6-dihexadecanoate and undecyl ester produced by each of *Lc. lactis* and *Lb. plantarum*. Also 2,3-propyl ester was produced by *Lb. plantarum*, while 4-pentadecyl ester and 3-pentadecyl ester were produced by mixed culture of these organisms with *Lactococcus lactis* at one-month. The aldehydes 2-tridecenal and trihydroxybenzaldehyde were each produced by *Lactococcus lactis* Gw-5 and *Lactobacillus plantarum* GSm-10 in single culture at one week and were later found to be undetectable at one-month of ripening.

There are a number of aroma attributes represented by organic acids, alcohols, ketones, esters and aldehydes in fermented dairy products. The type of flavour precursors produced is dependent on the metabolic pathways. For instance, hydrolysis of lipids produces fatty acids that are derivatives of aroma precursors such as lactones, esters and ketones but there are other pathways including proteolysis which yields amino acids from casein degradation, and glycolysis involving conversion of pyruvate to aroma ions (McSweeney and Sousa 2000; Van Kranenburg et al., 2002; Smit et al., 2004). Some of the flavour compounds and their precursors in ripened cheese are derived from butanoic acid, and lead to butter-creamy notes due to acetoin, fatty notes from 2-heptanol and cheesy-goat notes from hexanoic acid (Liu et al., 2022; Sarhir et al., 2019). Some of these compounds were detected in this study and would imply that producer organisms would lead to these respective notes in ripened Gouda cheese from which they were isolated. Studies reported by Jiang et al. (2020) and Garnier et al. (2020) showed that the most abundant volatile compounds in fermented dairy products including cheese are 2, 3-butanedione, acetone (ketones), hexanoic acid, nonanoic acid, octanoic acid, benzoic acid (organic acids), benzaldehyde (an aldehyde), dimethyl disulfide and ethyl acetate (ester), which is generally congruent with the results obtained from this study.

Lc. lactis is the primary starter culture used in production of Gouda cheese. It is possible that some of the *Lc. lactis* isolated in this study could be part of the cheese starter culture. However, it was evident from Table 7 and Figure 3 that a variety of many other non-starter microorganisms

including non-starter lactic acid bacteria (LAB) such as *Lb. plantarum*, spoilage and pathogenic bacteria were prevalent in the differently ripened Gouda cheese samples. The starter (*Lc. lactis*) and non-starter (*Lb. plantarum*) LAB produced a variety of aroma compounds under different treatments and ripening periods. For the production of Gouda cheese of consistent quality, entrepreneurs would need to invest in studies required to determine optimal ratios and types of some of the identified starter and non-starter LAB strains that are known to pose positive effects on cheese quality properties.

CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The study objectives were all achieved as indicated in the results section above and the following conclusions have been drawn from the study findings;

- A number of microbial populations including molds, yeasts and bacteria dominate the process of Gouda Cheese production. The study also confirmed that milk pasteurization was only effective at killing yeasts and molds but ineffective for heat-resistant (thermoduric) bacteria.
- Besides the starter culture (*Lactococcus lactis*) used during the production of Gouda cheese, the finished product contained a number of non-starter organisms including spoilage, pathogenic and non-starter lactic acid bacteria (NSLAB) dominated by, *Streptococcus lutetiensis* and *Lactobacillus plantarum.*
- *Lc. lactis* and *Lb. plantarum* isolated from Gouda cheese inhibited spoilage and pathogenic microorganisms which could enhance safety attributes of the cheese through various mechanisms such as acid and bacteriocin production. Each of the tested LAB genera inactivate Gram negative (*Salmonella* and *E. coli*) and Gram-positive (*Staphylococcus aureus*) bacteria on solid media assays. Activity was more pronounced against Gram negative than Gram-positive indicator bacteria. This effect was lost in liquid media assays implying that, *Lc. lactis* and *Lb. plantarum* isolated from Gouda cheese could only act as bio-preservatives in solid food matrices.
- *Lactobacillus plantarum* and *Lactococcus lactis* isolates had good growth properties in milk. However, *Lc. lactis* had better growth profile than *Lb. plantarum*. *Lc. lactis* had better acid production capacity than *Lb. plantarum*. Co-culture did not enhance acid production.
- Short term ripening of milk with *Lc. lactis* and *Lb. plantarum* enhanced aroma production in single culture, while long term ripening enhanced aroma production in mixed culture.

5.2 Recommendations

1. *Lc. lactis* isolated from Gouda cheese should be applied as primary starter culture to enhance curd formation owing to its high acid production capacity. The organism should also act as a bio-preservative, especially against undesirable Gram-negative bacteria, through acid production.

2. For prospecting commercial production of ripened fermented dairy products, 3 log CFU/ml of *Lc. lactis* and *Lb. plantarum* should be applied and incubated in a cold room for one week. The same effect should be attained by co-culture of these organisms and incubated in a cold room for one month.

3. Future research should investigate aroma production of the Gouda LAB isolates in real cheese model and other food matrices including plant-based products such as Bushera.

4. There is a need for more research efforts to probe aroma profile analysis of these organisms using more precise methods such gas chromatography olfactometry (GC-O).

5. Further studies should investigate the general hygiene of the cheese production facilities, workers and ripening conditions, among others, in order to establish the variations and sources of pathogenic bacteria recorded in the results in this study.

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