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Diversity of Bacterial Strains and Bacteriophages in Norwegian Cheese Production

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DIVERSITY OF BACTERIAL STRAINS AND BACTERIOPHAGES IN NORWEGIAN CHEESE PRODUCTION

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LIST OF ABBREVIATIONS AND ACRONYMS

Bp – base pair

BS – bulk starter

CaCL2 – Calcium chloride

CFU-Colony forming units

CO2 - Carbon dioxide

CWPS – Cell wall polysaccharides

DVI- Direct Vat Inoculation

dNTP -Deoxynucleotide triphosphates

dsDNA – double stranded deoxyribonucleic acid

ICTV - International Committee on Taxonomy of Viruses

g-gram

hr- hour

Kb-kilo base

LAB - lactic acid bacteria

MgCl2 – Magnesium chloride

mM – mili molar

NaOH – Sodium hydroxide

OD – Optical density

PCR - Polymerase chain reaction

Pp -page

RBPs - receptor-binding proteins

RNA - Ribonucleic acid

rRNA - Ribosomal ribonucleic acid

UPGMA - unweighted pair group method with arithmetic averages clustering algorithm

Φ - Phages

°C Degrees Celsius

µ - micro

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ABSTRACT

A study on diversity of bacterial strains and bacteriophages in Norwegian cheese production was assessed on samples from TINE`s cheese production. Samples were obtained from a dairy plant in Nærbø that was experienced fermentation problems. 96 bacterial strains were also isolated from another TINE's dairy plant in Storsteinnes which was producing quality cheese. Both dairies were using the same commercial starter cultures. For phage isolation bulk starter and whey samples were used to infect 96 bacterial strains. Forty phages were isolated from specific indicator strains. Using the isolated phages, phage typing of all the 96 strains was performed, and plaque formation was obtained on 80 strains. The sensitivity of bacterial strains was highly variable and isolated phages had a broad host range infecting between 4% (Φ837) and 44% (Φ878) of the strains. In addition, $(GTG)_{5\text{-}PCR}$ enabling the differentiation of all the bacterial strains in to 8 clusters. Furthermore, cell wall polysaccharide genes typing of bacterial strains were also carried out and 91 strains were found to have genes encoding different CWPS types, while five strains were suggested to have another type of cell surface. Our results showed that *Lactococcus lactis* of CWPS group B were the predominant starter culture used in the Norwegian cheese production. Finally, the effect of the isolated phages in fermentation activity of mixed starter cultures was also assessed and results showed a change in pH during milk fermentation. Though, pooled phage isolates caused inhibition in acid production, our phage isolates alone were not shown to cause a severe effect in the activities of the commercial starter culture. Therefore, further studies using single starter culture are recommended to reveal the actual impact of the isolated phages on dairy fermentation processes.

1. INTRODUCTION

The dairy industry is one of the most important food industries that involves growth and metabolic activities of lactic acid bacteria (LAB). The uses of LAB as starter cultures have also great industrial significance due to their crucial role in the manufacturing, flavor, and texture development of fermented dairy foods (Cogan *et al*., 2007; Shiby and Mishra, 2013). The production of lactic acid and other metabolites by LAB starter cultures in the food industry are very important for ensuring high-quality products (del Rio *et al*., 2012; Marco *et al*., 2012) and control of pathogenic and spoilage microorganisms (Carminati *et al*., 2010). Several fermented food products, including cheese, fermented milks, and cream butter are highly acceptable by consumers because of their flavor, aroma and high nutritive value (Marco *et al*., 2012; Shiby and Mishra, 2013). Moreover, much research has been done on the health benefits associated with the consumption of cultured dairy foods and probiotics, particularly their role in modulating immune function (Cogan *et al.*, 2007). Other health benefits of fermented milk products include prevention of gastrointestinal infections, anti-tumor activity, good for lactose intolerance cases, reduction of serum cholesterol levels and anti-mutagenic activity (Shiby and Mishra, 2013).

According to Hati *et al*. (2013), a starter culture is defined as "an active microbial preparation, deliberately added to initiate desirable changes during preparation of fermented products". It can be a single strain type or a combination of different strains, usually strains of *Lactococcus lactis* (*L. lactis*), *Streptococcus thermophilus (Str. Thermophilus)*, *Leuconostoc*, and/or *Lactobacillus* species (Garneau and Moineau, 2011). Strains of *L. lactis* and *Str. thermophilus* are commonly used starter cultures in the dairy industry (Deveau *et al.*, 2006). The genus *Leuconostoc* together with *L. lactis* subsp. *lactis* biovar *diacetylactis* are important flavor producers (Kleppen *et al*., 2012). These bacterial strains produce diacetyl, the source of aroma and flavor compounds in different dairy products, such as buttermilk, butter, and various cheese types (Bjorkroth and Holzapfel, 2006).

Bacteriophage infections during dairy fermentation processes have been a major concern for many years. Phages are obligate parasites of bacteria that attacks LAB starter cultures, and become the major sources of fermentation failure (spoilage or delay) causing economic losses on many dairy products (Moineau and Lévesque, 2005; Garneau and Moineau, 2011). Though, the problem of phages is well known in the dairy industry, there are also reports in the food, chemical, pharmaceutical, feed and pesticide industries (Emond and Moineau, 2007). Thus, understanding LAB phage-host interaction has paramount importance in finding solution for huge losses (Hassan and Frank, 2001; Deveau *et al.*, 2006).

Most known phages have dsDNA packaged into a capsid connected to a tail (Ackermann, 2007). All known phages infecting LAB members are classified under tailed phages (Marco *et al*., 2012). The tail of a phage is an essential structure both for phage classification and for host recognition. Although, tailed phages are classified in to ten species, the 936, c2, and P335 species are the most frequently isolated lactococcal phages from dairy environments (Deveau *et al*., 2006).

Starter cultures contains gram positive LAB and the composition of their surface structure contain cell wall polysaccharides (CWPS). The gene cluster encoding CWPS biosynthesis in various *L. lactis* strains contain highly conserved regions but exhibits genetic diversity, suggesting that CWPS structure could be a variable character between strains (Mahony *et al.,* 2013b). Although, the taxonomy of *L. lactis* has changed many times, currently it is based on phenotypic methods (Schleifer *et al.,* 1985; Rademaker *et al.,* 2007) and includes two subspecies *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris,* which correlate with its genotype.

Lactococcal phage infections requires the recognition of receptors on the bacterial cell surface by receptor-binding proteins (RBPs) that are part of the phage tail structure (Kleppen *et al*., 2012; Kelly *et al*., 2013). However, binding of phage to the cell surface is a very specific process (Holmfeldt *et al.,* 2007). This specificity of RBPs can be partly due to the specific CWPS that can serve as host receptor for phage particles (Ainsworth *et al*., 2014), accessibility of bacterial receptor and physiochemical properties of the cell envelope (Ray and Bhunia, 2013). Recent studies indicated that *L. lactis* has three distinct genetic loci for CWPS biosynthesis termed the A, B, and C types (Mahony *et al*., 2013b; Ainsworth *et al*., 2014), which can be linked to the RBP phylogeny of 936 phages (Mahony *et al*., 2013b).

Further knowledge on the nature of CWPS-types will allow selection of dairy starters and design starters resistant to certain groups of phages with known RBPs, taking this into account they can be used in strain rotation to prevent phage attacks (Ainsworth *et al.,* 2014). Since, rotation of various phage-unrelated starter cultures is helpful to avoid recurrent amplification of the same phage over consecutive fermentation processes (Garneau and Moineau, 2011; Kleppen *et al.,* 2011). In dairy production, 'bulk starter' in liquid form was used to inoculate the milk used in the manufacture of cheese, yoghurt, buttermilk and other fermented products (Marco *et al.,* 2012). However, the use of bulk starter in cheese making is an advantage for the phages, since this procedure gives phage with an opportunity to multiply prior to fermentation in the production vats (Kleppen *et al.,* 2011). Furthermore, phages may remain in the whey

protein concentrate (liquid or dried) and contaminate the products to which it is added (Chopin, 1980). Nowadays, the use of starter cell concentrates designated as Direct Vat Inoculation (DVI) cultures have increasing being used in cheese manufacture, this can serve as the only inoculum in fermentation vats and avoid fermentation problems caused by phages in bulk starter (Kleppen *et al.,* 2011).

To control phage infection, culture rotation is one of the methods used in dairy production processes. However, culture rotation can`t eliminate phage growth in cheese milk in vats, but if phage numbers are reduced to below 10,000 PFU/mL of cheese whey, acid production will not be affected (Huggins, 1984). Thus, it is important to determine the sensitivity of starter bacteria to the phages isolated from the dairy. This study was carried out with 96 bacterial strains isolated from a BS obtained from TINE`s good dairy plant in Storsteinnes. Bulk starters and whey samples were collected from another TINE`s dairy in Nærbø, that had fermentation problem in the cheese making processes using the same starter culture. Therefore, our objectives were to investigate the diversity of bacterial strains from the starter culture that were producing quality products in Storsteinnes and to determine diversity of phages in the samples from Nærbø. These samples were tested for the presence of phages specific to the 96 strains. In this study plaque assays and PCR methods were used with the following specific objectives:

- To isolate phages from bulk starter and whey samples
- To determine the phage susceptibility of start cultures strains
- To investigate relationship between phage host range and diversity of bacterial strains
- To compare PCR methods with phage typing for differentiation of LAB strains
- And possibly to identify phages affecting acidification process

2. LITERATURE REVIEW

2.1 The process of cheese making

Cheese making is a process in which the conversion of liquid milk into a solid mass of cheese is done via coagulation (or precipitation) of milk protein. Milk, contains about 3.3% proteins. Of the protein portion, about 80% is casein and the remaining 20% are known collectively as whey proteins. According to Hutkins (2006), there are three ways of milk coagulation. First, milk can be coagulated by acids produced by LAB. When the milk pH reaches 4.6, casein is at its isoelectric point and its minimum solubility, and therefore it precipitates. It is important to realize that casein coagulates at pH 4.6 whether acidification occurs via fermentation generated acids or simply by addition of food grade acids direct into the milk. The second and most common way to effect coagulation is by the addition of the enzyme chymosin (or rennet). This enzyme hydrolyzes a specific peptide bond located between amino acid residues 105 (a methionine) and 106 (a phenylalanine) in casein proteins (Kosikowski and Mistry, 1997; Hutkins, 2006). Besides the protein separation, the fermentative action of specific LAB stains may lead to an extended bacterial fermentation and removal of toxic or antinutritive factors, such as lactose and galactose from fermented milks to prevent lactose intolerance (Hati *et al.,* 2013).

In contrast to acid-precipitated casein, the coagulated casein network formed by chymosin treatment traps nearly all of the milk fat within the curd. Most of the cheeses manufactured around the world rely on chymosin coagulation. However, it is worth emphasizing that even though chymosin, alone, is sufficient to coagulate milk, lactic starter cultures are also absolutely essential for successful manufacture of most hard cheeses (Hati *et al.,* 2013). The lactic acid bacteria that comprise cheese cultures not only produce acid and reduce the pH, they also contribute to the relevant flavor, texture, and other properties of cheese (Hutkins, 2006). Finally, it is possible to form a precipitate by a combination of moderate acid addition (pH 6.0), plus high heat (>85°C).Whey proteins are denatured under these conditions, thus the precipitate that form consists not only of casein, but also whey proteins.

Another aspect of LAB that affects cheese production is autolysis (Lortal and Chapot-Chartier, 2005). Autolysis of the starter cells is followed by the release of intracellular peptidases in the curd. Bacterial autolysis is a result of the regular degradation of peptidoglycan by autolysin activity paired with the cessation of peptidoglycan synthesis induced by unfavourable environmental conditions and consequent cell lysis. Furthermore,

genetic strategies exist for the induction of phage holins and lysins to promote lysis of the starter cells during maturation (Hati *et al.,* 2013).

2.2 Lactic acid bacteria as starter cultures in cheese production

Commercial dairy starter cultures are generally composed of lactic acid-producing and citric acid-fermenting lactic acid bacteria. Although commercial dairy starters are available as cultures which can be added directly to milk without any intermediate transfer or propagation, it is a common practice that cheese makers produce in-house bulk starters. A bulk starter is defined as starter cultures contain carefully selected strains propagated at the dairy plant for inoculation into milk (Elsborg *et al.,* 2001). Such bulk starters are generally made by inoculating heat treated milk with a volume of a previous bulk starter or with a freeze-dried or frozen starter culture preparation, followed by incubating the inoculated milk under conditions permitting the starter culture strain(s) to propagate for a sufficient period of time to provide a desired cell number (Elsborg *et al.,* 2001; Lortal and Chapot-Chartier, 2005).

During cheese maturation, several aromatic compounds are generated due to the action of endogenous milk enzymes as well as the proteolytic and lipolytic activities of LAB present in the cheese (Hati *et al.,* 2013). They begin the process by degrading proteins in the starting material. The proteases of the starter culture, as well as the rennet in curd and the plasmin in milk, degrade proteins, namely casein, to produce free amino acids and peptides (Smit *et al.,* 2000). These amino acids are essential precursors to the volatile aroma compounds that are responsible for the distinct cheese flavors (Yvon *et al.,* 1997).

2.3 Types of starter cultures and selection criteria for suitable strains

The production of fermented dairy products such as cheese-making is based on LAB ability to ferment lactose causing a speedy acidification of milk through lactic acid production, which decrease the pH, consequently affecting cheese production process and eventually cheese composition and quality (Briggiler-Marco *et al.,* 2007). There are about 11 genera of LAB, but in dairy starter cultures the commonly used are strains of four genera namely *Lactococcus*, *Leuconostoc*, *Lactobacillus* and *Streptococcus* (Hassan and Frank, 2001; Hati *et al*., 2013). Strains of *L. lactis* and *Str. thermophilus* are the most actively working starter culture bacteria in the dairy fermentation industry globally (Deveau *et al*., 2006), whereas strains of *Lactobacillus* and *Leuconostoc* species are commonly used as adjuncts (Nieto-Arribas *et al*., 2010). Starter cultures can be used as single strain, mixed strain or multiple strains depending upon the desire what type of products to be prepared (Fernandes, 2008). Mixed

starter cultures may be composed of different genera, species, and strains of LAB which together form a complex culture (Hassan and Frank, 2001).

In selection of LAB starter culture strains, phenotypic methods based on physiological or biochemical characteristics have been widely used (Kongo *et al*., 2007). These phenotypic methods are very important in finding functional starter cultures which may lead to an improved fermentation process and enhanced quality of the end products (Hati *et al*., 2013). However, due to the presence of huge number of LAB strains with similar phenotypic characteristics, in most cases these tests are insufficient for accurate strains identification (Temmerman *et al*., 2004). Furthermore, the success of using functional starter cultures in a particular food has been shown to be strongly strain dependent (Hati *et al*., 2013). On the other hand, genotypic methods have much better discriminatory power in differentiation of all individual strains (Prabhakar *et al*., 2011). Hence, combination of both phenotypic and genotypic identification methods is preferred approach (Temmerman *et al*., 2004, Hati *et al*., 2013).

Based on their physiology and growth characteristics starter culture can be classified as mesophillic starter cultures (optimal growth temperature between 25-30°C) or those that have optimal growth temperature between 37ºC - 45°C are known as thermophilic starter cultures (Cogan, 1996; Fernandes, 2008). Similarly, their biochemical characteristics can also be classified either as homofermentative or heterofermentative (Ray and Bhunia, 2013).

2.3.1 Mesophilic starter cultures

The microorganisms under this category include mainly strains of two genera *Lactococci* and *Leuconostoc* (Fernandes, 2008**)**. These mesophillic starter cultures are used in the production of many cheese varieties mainly because of their acid producing activity, gas production, and production of enzymatic activity for cheese ripening (McSweeney, 2004). In complex cultures, the flavour-production is either exclusively by *Leuconostoc* spp. or by combination of *Leuconostoc* and *L. lactis* subsp. *lactis* biovar *diacetylactis* strains (Parente and Cogan, 2004; Kleppen *et al*., 2012).

According to Lodics and Steenson, (1993), the nature of mixed- culture strain mesophilic starter cultures can be grouped by their composition and are stated as:

O-type: starter cultures consist of *Lactococcus* spp., the main homofermentative LAB, which do not ferment citrate; this type includes *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*.

D-type: in addition to the O-type starter cultures, these cultures contain a flavour producing LAB, called citrate-fermenting lactococcus known as *L. lactis* subsp. *lactis* biovar *diacetylactis* (Kleppen *et al*., 2012; Tamara, 2013). In addition to production of diacetyl, it

also produces carbon dioxide which contributes a delicate flavour. **L-type**: starter culture contains *Leuconostoc* and *Lactococcus* spp that do not ferment citrate. In addition to the O-type bacteria, this includes *Leuconostoc* spp. as the main flavour producing bacteria. It produces diacetyl acetic acid, acetaldehyde and other flavour compounds but less carbon dioxide than the D-type. And **LD-type**: These contain combination of cultures contain *Leuconostoc* spp. as well as lactococci found in D cultures. This includes cultures of *L. lactis* subsp. *lactis* biovar *diacetylactis* and *Leu. mesenteroides* subsp. *metesenroid* give a fine mixture of dedicated flavour and aroma (Hassan and Frank, 2001; Deveau *et al.,* 2006).

In dairy fermentations, strains of *Lactococcus lactis* (*L. lactis*) are the most extensively known mesophilic bacteria used as starter culture during the manufacturing of various fermented products, including cheese, buttermilk, and sour cream (Hassan and Frank, 2001; Deveau *et al*., 2006; Marco *et al*., 2012). The taxonomy of *L. lactis* has changed repeatedly and still is confusing in some aspects (Tamara, 2013); previously it was known as lactic streptococci, however, since 1985 it has been placed in the new *Lactococcus* taxon (Schleifer *et al.*, 1985). The current taxonomy of *L. lactis* is based on phenotype and includes four subspecies (*lactis*, c*remoris, hordniae,* and the newly identified subsp. *tructae*) and one biovar (subsp. *lactis* biovar *diacetylactis*) (Rademaker *et al.,* 2007). Among these, *L. lactis* subsp. *hordniae* and subsp. *tructae* have never been isolated from dairy products (Tamara, 2013). Thus, in dairy production *L. lactis* species has two subspecies, *L. lactis* subsp. *lactis* and *L. lactis* subsp. c*remoris (*Fernández *et al*., 2011). Moreover, various molecular methods including 16S rRNA and gene sequence analysis of *L. lactis* isolates of dairy have found the existence of two main genotypes that have also been called *L. lactis* subsp. *lactis* and *L. lactis* subsp. c*remoris (*Tamara, 2013). The *L. lactis* subsp. *lactis* is more heat and salt tolerant than *L. lactis* subsp. *cremoris* and has variant of *L. lactis* (*L. Lactis* subsp. *lactis* var. *diacetylactis*), which converts citrate to diacetyl, carbon dioxide, and other compounds (Cogan and Jordan, 1994; Hassan and Frank, 2001). Both subsp. are homofermentative; more than 95% of their end product from lactose is lactic acid (Hassan and Frank, 2001).

Leuconostocs like other LAB are gram-positive, have coccoid shape, and are used in the dairy industry to produce diacetyl, carbon dioxide, and acetate from citrate (Cogan and Jordan, 1994; Hassan and Frank, 2001; Kleppen *et al*., 2012). Leuconostoc strains are important flavor producers both in the L-type and during DL-type mesophilic starters together with *L. lactis* subsp. *lactis* biovar. *diacetylactis* (Kleppen *et al*., 2012). The species and subsp. of this genus that are mainly associated with dairy starter cultures are *Leuc. mesenteroides* subsp. *cremoris* (previously, *Leuc. citrovorum*) and *Leuc. lactis* (Hassan and Frank, 2001; Huys *et*

al., 2011). These species are distinguished from other LAB by being mesophilic heterofermentative cocci (Hassan and Frank, 2001). In addition, a significant amount of CO_2 is produced from their citrate metabolism and heterofermentative lactic acid fermentation (Ali *et al*., 2013). This CO_2 production is important for eye formation and the texture of semi-hard cheeses (Hassan and Frank, 2001; Cantor *et al*., 2004); moreover, CO_2 production is crucial for the opening of the matrix of white- and blue-molded cheeses (Cantor *et al.*, 2004).

2.3.2 Thermophilic starter cultures

These types of cultures consist of a mixture of *Str. thermophilus* and *Lactobacillus* species *(Lb.* spp.), usually *Lb. helveticus*, *Lb. delbrueckii* subsp. *bulgaricus*, or *Lb. delbrueckii* subsp. *Lactis* (Hassan and Frank, 2001). The only Streptococcus sp. which is important in dairy fermentation is *Str. thermophilus*, and mostly it is used in combination with other starter cultures for the production of cheese (Swiss and Italian varieties), yogurt, and fermented milk products (Tamime, 2002). These cultures are generally employed in the production of yoghurt, acidophilus milk, to produce Italian and Swiss cheese varieties and yogurt (McSweeney, 2004). The starter culture responsible for the development of the typical Swiss cheese must survive the high temperatures (50–52^{o}C) in order to exert its effects in flavor production and eye formation (Tamime, 2002). Swiss cheese is unique due to the interactions between the three major bacteria that occupy it: *Lb. casei, Str. thermophilus*, and *Propionibacterium shermanii* (*P. shermanii*). *P. shermanii* is responsible for producing the holes and the distinct flavor of Swiss cheese. Because it metabolises lactic acid to produce carbon dioxide and propionic acid. The gas is needed for the formation of the characteristic eyes in the cheese, and the propionic acid contributes towards the sweet, nutty flavour of these cheeses (Fernandes, 2008). The growth rate of the bacterium is dependent on the surrounding temperature, pH, and bacteria. The optimal growth for *P. shemanii* is in warm temperature and at a pH of 5.3. The bacterium's growth is also dependent of the availability of lactic acid which is produced by *L. helveticus* and *S. thermophilus* (Kurtz *et al.*, 1959).

2.4 Phages of LAB and their effects

Bacteriophages also called phages are the most abundant biological units on earth that infect and use bacterial resources (Garneau and Moineau, 2011; Marco *et al.*, 2012). Their number is estimated to be between 10^{30} to 10^{32} total phage particles which are estimated to be 10-fold of the total bacterial numbers (Marco *et al*., 2012). The effectiveness of dairy starter cultures can be affected by many factors such as by the quality of raw milk, presence of antibodies in milk, presence of antibiotics residues, bacterial interactions, and phages (Surono and Hosono,

2011). Moreover, Kleppen and his coworkers (2012) have been indicated that bulk cultures infected with phages contamination in the Vat can disseminate phages during fermentation process. Virulent phages of *L. lactis* strains are the most frequently encountered phages in dairies during cheese and dairy beverages production. Thus, lactococcal phages have been the focus of research interest over the past three decades, because they have been reported to cause economic losses in dairy industry (Kelly *et al*., 2013). Many research findings confirmed that three lactococcal phage species: 936 type, P335, and c2 are the most common isolated phages responsible for dairy fermentation losses (Kleppen *et al*., 2011; Marco *et al*., 2012; Mahony *et al*., 2012a; Ray and Bhunia, 2013; Mahony and van Sinderen, 2014). However, the most globally prevalent lactococcal phages in dairy industries is the 936-type, and those phages cause the highest threat to this industry (Mahony *et al*., 2012a).

As Leuconostoc strains do not have significant role in lactic acid production in milk, phage infections of this starter cultures may remain undetected during processing but will be detected in the final products (Marco *et al*., 2012). Thus, the effect of phage infection in *Leuconostoc* spp. is directly related with the poor qualities of dairy products, which are resulted from the reduction in the concentration of aromatic compounds or in CO_2 production (Hemme and Foucaud-Scheunemann, 2003).

2.5 Lactic acid bacteria phages- morphology and classification

Phages have double-stranded or single-stranded DNA or RNA. A phage contains several proteins (that make the head, tail, tail fiber, and contractile sheath) and DNA, which can be linear or circular (Ray and Bhunia, 2013). Most phages (96%) isolated so far belong to one taxonomic order of *Caudovirales (*Ackermann and Kropinski, 2007; Marco *et al*., 2012). Bacteriophages within this order contain tails and a linear dsDNA genome. They are further classified into three phylogenetically linked families of: *Myoviridae, Siphoviridae, Podoviridae (*Ackermann and Kropinski, 2007; Deasy *et al.,* 2011). *Myoviridae* phages contain a long and contractile tail, while *Siphoviridae* and *Podoviridae* are equipped with a non-contractile tail, long and short, respectively (Ackermann, 2003; Mahony *et al.,* 2012b; Ray and Bhunia, 2013). The round (prolate) or hexagonal (isometric) dsDNA molecule is packed in the head. Based on the nature and size of the phage head structure, phages of different families can be grouped in to three morphotypes, namely morphotype type 1, which has small isometric heads, morphotype type 2 with small prolate heads, and type 3 characterized with large prolate heads (Ackermann, 2007; Kleppen *et al*., 2012; Ray and Bhunia, 2013).

Classification of lactococcal phages has been proposed in to diverse classification schemes (Deveau *et al*., 2006; Marco *et al*., 2012). Phage DNA is enclosed by head proteins, while the tail section has variable lengths and is used to attach to their host receptors, through which DNA passes into bacteria (Hassan and Frank, 2001). Moreover, phages classification by the International Committee on Taxonomy of Viruses (ICTV) is based on different parameters including morphologies and their genome similarities (Deveau *et al*., 2006). Conversely, all phages infecting *Str. thermophilus* show similar morphology with long, non-contractile tails and isometric capsid arrangements of the *Siphoviridae* family (Mills *et al*., 2011). Such classification of phage is very important in identification process of those abundant biological natures, and is mainly based on their morphological, serological, and DNA-DNA homology characteristics (Mahony and van Sinderen, 2014).

2.6 Determination of host specificity in phage infection

Phage infections are caused by either virulent or temperate phages. Infection with lytic (virulent) phages results in release of infectious viral particles (virions) into the environment, whereas temperate phages integrate their DNA into the host chromosome and do not immediately produce new virions (Hassan and Frank, 2001). Phage infections initiates when the RBPs located on the phage tail structure are recognized and make an attachment to receptors sites on the bacterial cell surface (Kleppen *et al*., 2012; Kelly *et al*., 2013). This can`t happen in every bacterium but bacteria with specific receptor sites can only serve as host for phage particles. The presence of such sites determines the specificity of a particular phage (Hassan and Frank, 2001), thus phages are host specific, but this can be between one specific strains for a specific phage to several related strains for a phage (Holmfeldt *et al.*, 2007). On the other hand, a bacterial strain can also be the host of many different types of phages. On the contrary, a bacterial strain can have restriction enzymes that can hydrolyze and destroy the DNA of a phage. Furthermore, though phages can be lytic or temperate, most phages require Ca^{2+} for their adsorption on the cell surface of starter cultures bacteria (Ray and Bhunia, 2013).

3. MATERIALS AND METHODS

3.1 Origin of samples and bacterial strains

96 bacterial strains isolated from cheese making process at TINE's dairy plant in Storsteinnes (Northern Norway), which had no fermentation problem was kindly provided by Cyril Frantzen. These strains were isolated from samples that were collected 1hr after BS of a commercial starter culture S (hereafter, called Culture S), was added to the fermentation Vat, and collected samples were diluted and grown on LM17, then 96 strains were isolated and used in this study. The 96 isolated strains were named plate number 8; and were maintained both at -80°C or -20°C. Three bulk starter samples and three whey samples were obtained from another TINE's dairy plant in Nærbø, where a fermentation problem was reported. In both sites, culture S was used for fermentation process. Thus every practical related to this study was based on the same starter culture S. The samples were used to detect phage diversity in that dairy.

3.2 Phage isolation and differentiation

3.2.1 Phage isolation by plaque assay method

Materials:

LM17-Agar	LM17 broth	Microtiter plate reader
Microbiological Agar	Lactose	Centrifuge
Balance	Spatula	Centrifuge
Measuring cylinders	Petridishes	water bath
250 ml size Bottles	Distilled water	Eppendorf tubes
5mM $CaCl_2$ solution	Culture tubes	Microwave oven
Incubator 30ºc	Test tube rack	well-stamper
96% Ethanol	96 well microtiter plate	

3 bulk starters and three whey samples collected from Tine`s cheese production in Nærbø; and this center has been reported to have fermentation problems. The commercial starter culture in this dairy was the `S` type which is the same as starter cultures used in Storsteinnes.

96 LAB strains isolated from a cheese making process at Storsteinnes

Procedures:

i. First, bulk starter and whey samples were centrifuged; supernatants were collected and filtered using sterile 0.45 µm pore size filter.

ii. For phage detection from bulk starter and whey samples, a six fold dilution steps of both samples was prepared to be used as a source of phages.

iii. Using sterile toothpicks bacterial strains from -20°C were transferred to 4 ml of LM17 broth (M17 broth supplemented with 0.5% lactose) and after overnight growth at 30°C, 10 µL of this was transferred to 3ml of another LM17 broth and incubated until their exponential growth phase (OD-value ≈ 0.2) was reached.

iv. Then 2.5ml of LM17 molten soft agar (LM17 broth containing 0.8% agar) supplemented with 5 mM $CaCl_2$ was kept in water bath (46°C), this was used to mix with 500 µL of host cells grown to high cell density (OD value ≈ 0.2).

v. The resulting suspension was then immediately poured on top of LM17 Agar to form a thin layer which hardens and immobilizes the bacteria.

vi. Using 48 well stamper, approximately 5µL suspensions of six step serial dilutions of all samples (from step 2): Whey K1 19/7, Whey K25, BS 19/7, BS 18/7, BS 17/7, and Whey were plated on to LM17 Agar and incubated at 30°C.

vii. After overnight incubation, plates were examined for lysis zones/ plaque formation against each producer, when bacterial growth was poor results were kept until 72 hours of incubation, before they discarded as negatives. After all 96 LAB strains were exposed with six whey and bulk starter samples, 40 bacterial strains were found to form least one plaque from the six samples.

3.2.2 Propagation of phages

When 40 out of the 96 bacterial strains gave plaque formation, the plates with plaques were used to propagate and purify phages, by taking one isolated plaque per host strain and amplify on their own respective bacterial hosts. The 56 `non-plaquing strains` meaning strains that were stamped with bulk starter and whey samples but didn`t give plaques were also scratched from the stamped surface and used to indicate the presence of phages.

Materials:

Sterile membrane filters 0.45 µm pore-sizes

40 plaque containing plates/strains

56 non-plaqueing strains

Procedures:

i. Isolated plaques (from 3.2.1) were picked and transferred to infect their respective hosts while they were exponentially growing in tubes with 10mL of LM17 broth supplemented with 5mM $CaCl_2$ solution.

ii. From the 56 strains that were previously stamped with bulk starter and whey samples but failed to form plaques, their surface spots were used to enrich culture of their respective strains in 10mL of LM17 broth supplemented with 5mM $CaCl_2$ solution.,
iii. Tubes were incubated overnight at 30ºC, and checked for growth inhibition by comparison with tube which had only bacterial culture
iv. Phages propagated on their respective host strains were observed by the relative clearness of the broth, and were sterilized by filtration through 0.45 μm pore-size filters and stored at 4ºC for further assays.

3.2.3 Phage typing of strains

To determine phage host range and the bacterial susceptibility to specific phages, phage typing was conducted using phages isolated from plaques and with non-purified phages obtained from the non plaqueing strains. Similar to the previous (3.2.2) the same materials were used but the forty purified phages were applied at a time on each indicator strain. In addition, phage typing of the non-plaqueing strains was carried out using forty non-purified phages. In both ways, using a well-stamper, phage suspensions were applied on a lawn of host bacteria on top of LM17 agar, and plates were examined for cell lysis after overnight incubation, and when the bacterial growth was slow follow up was continued up to 72 hrs. Within this incubation periods, infection was considered severe if any sign of lysis (plaque formation) was seen in those tests. Such tests were used to determine host susceptibility and specificity of phages to their hosts.

3.2.4 Fermentation inhibition test

Acid production can be used to evaluate milk fermentation and is therefore an important method to test the activity of starter cultures. Parallel experiments were done on an overnight grown culture “S” using 10% skimmed milk. These cultures were treated with pooled phages. The addition of phage samples to the overnight culture was to estimate the impact of the isolated phages on fermentation activity of the starter cultures. Then various inhibition levels of lactose fermentation by the starter culture was reflected by the reduction of acid production measured over 4 hr incubation period at a temperature of 30ºC.

Materials:

10% Skimmed milk,	Mixed starter culture	Erlenmeyer flask.
Test tubes	pH meter	Sterile distilled water
Phages	Sterile filter 0.45 μm pore-sizes	

Procedures:

i. Preparation of 10% skimmed milk medium using powdered skim milk.

ii. In order to avoid other microbial contaminants in this skimmed milk, it was pasteurized at 95^{o}C for 30 minutes.

iii. This fermentation inhibition test was carried out in duplicates. The test scheme shown (Table 1), duplicate experiments where each tested culture had 2 control tubes and 5 treatment test tubes.

iv. First each tube was filled with 10ml of the pasteurized 10% skimmed milk.

v. Then Culture S was inoculated in to test tubes 2 to 7 with a concentration of 0.1% and strains were grown overnight at 22^{o}C.

vi. Another corresponding tubes containing 5ml of the 10% skimmed milk were prepared

vii. Control tubes received 3% of overnight bulk starter culture, while the other respective test samples (3-7) received 3% of same culture and 25µl of phage-lysates of each mixture (Mix 1 to 5).

viii. All samples were incubated at 30^{o}C for 4 hours and were determined by measurement of pH value of all test samples using a pH meter.

ix. Analysis of fermentation activity was performed in all treatment groups and was expressed as: pH of blank sample - pH of sample.

Table 1. Experimental set up for fermentation inhibition test

Test sample	pH1	pH2	pH change	Description
1	Blank	Blank	Blank	Milk (blank)
2	Control	Control	Control	Control (contain bacteria only)
3	Mix 1	Mix 1	Mix 1	contain mixture of six broad host spectrum phages
4	Mix 2	Mix 2	Mix 2	contain mixture of 21 mainly narrow-spectrum phages
5	Mix 3	Mix 3	Mix 3	contains 40 phages obtained from the plaque forming strains
6	Mix 4	Mix 4	Mix 4	contain 39 phages (from Cyril Frantzen)
7	Mix 5	Mix 5	Mix 5	Contain 79 phages (Mix 3 and 4)

In this experiment, the tests were carried out in duplicate and pH measurement was done and average value was used to calculate the bacterial activity. The Blank samples indicate tubes which contain neither bacterial culture nor phages, but only milk sample used as a reference for evaluating the treatment groups. The control group contain bacterial culture only.

3.3 Differentiation of bacterial isolates

3.3.1 Growth inhibition test

To determine the phage sensitivity, all the 96 bacterial isolates that were kept at -80°C were grown in LM17 broth. Cultures were reactivated by sub culturing in another fresh media; half of the plate was left as negative control and the second half received approximately 5µL of bulk starter as a source of phage. Growth was determined after 16 hr by microtiter plate reader.

Materials:

96 microtiter plate Phage lysate

Microtiter plate reader 96 strains

5 mM $CaCl_2$ solution

Procedures:

i. Using a 48 well stamper, 48 strains were transferred to new microtiter plate containing 180 µL LM17 broth and incubated at 30°C for two days.
ii. After appreciable growth was obtained, sub-culturing to another plate with the same procedure was done; within 2-3 hours of incubation at 30°C, OD-value ≈ 0.2 was reached.
iii. At this time one group of the 48 duplicated strains were exposed to bulk starter 19/7 (BS19/7) at 5% concentration of the BS19/7 and 5mM $CaCl_2$ solution, and the other half plate used as a control (Appendix 3).
iv. Then growth kinetics study was conducted using microplate reader through 16 hours of incubation time.
v. With the same procedure the other 48 strains were also tested separately.

3.3.2 DNA preparation for genotypic differentiation of strains

Materials:

LM17 broth 30ºC Incubator

96 bacterial strains sterilize distilled water

Test tubes lysis buffer (0.25 % sodium dodecyl sulfate + 50 mmol/L and NaOH)

PCR tubes PCR Machine to incubate cell for DNA extraction

Eppendorf tubes Centrifuge for collection of bacterial cells

Procedures:

i. All strains were grown overnight at 30ºC in 5ml of LM17 broth and centrifuged twice by washing at intervals, and then re-suspended in 100µL of sterilized distilled water and kept at -20ºC.
ii. Bacterial DNA was isolated by alkaline extraction method with some modification to the procedure described by Svec and Sedlacek,(2008). Briefly, 25µL of the 100µL stored cells were centrifuged and 20µL of the supernatant part was removed.
iii. The remaining 5µL was resuspended with 20µL of lysis buffer (0.25 % sodium dodecyl sulfate + 50 mmol/L and NaOH) and heated at 95ºC for 15 minutes.
iv. After extraction, the resulting cell lysate was diluted by adding 180µL of sterilized distilled water and maintained at -20ºC to be used by PCR reactions.

3.3.3 Separation of bacterial strains by $(GTG)_5$-PCR fingerprinting

The $(GTG)_5$-PCR fingerprinting method was used to detect and differentiate bacterial isolates. After genomic DNA of all strains was extracted, PCR amplification with broad specificity of $(GTG)_5$-primer (5´-GTGGTGGTG GTGGTG-3´) was performed. The PCR amplification was carried out with slight modifications of Svec and Sedlacek (2008) protocol as described below:

Materials:

1.5% Agarose gel	0.5x TAE buffer	1kb ladder
Gelred (2000 times diluted stock solution)	6x loading buffer	Volt-meter
PCR machine (Thermal Cycler)	Distilled water	-20ºC Freezer
Gel-electrophoresis equipments	NanoDrop ND-1000	+4ºC refrigerator
(rack, molding form, comb)	30ºC incubator	
Used primer:-5`-GTGGTGGTGGTGGTG-3`	Gel photo system with UV spectrum	

Table 2. Procedure for master mix preparation

Master mix reagents	Amount/sample	24 samples at a time (24x)
10mM PCR buffer	1µL	24µL
10mM dNTP	1µL	24µL
50mM $MgCl_2$ solution	5µL	120µL
Taq-DNA Polymerase	0.5µL	12µL
Template DNA (bacterial cell)	1µL	24µL
$(GTG)_5$ primer	5µL	120µL
Adjust volume to 50µL with sterile dH2O	36.5	876
Total volume (µL)	50	1200

Procedures:

i. Based on table 2, the volume of the PCR mixture was adjusted to 50 µl with sterile distilled water
ii. PCR amplification was performed in MyCycler^{TM}, thermal cycler at the following conditions; first denaturation step of 7 min at 95^{o}C, followed by 30 cycles of 95^{o}C for 30 seconds, 45°C for 1 min, 65^{o}C for 1min and a final elongation step has been programmed at 72°C for 7 minutes.
iii. PCR products were separated by electrophoresis on 1.5 % agarose gels for 4 h at 100V in 0.5× TBA buffer. Using 1Kb DNA ladder (BILABS 1kb Ladder # 32325), PCR fingerprints were digitized and analyzed with the band pattern analysis software package, GelCompar II (Applied Maths, Kortrijk, Belgium).

3.3.4 CWPS genes typing of strains by multiplex PCR

Differentiation of bacterial strains based on detection of genes that encode different CWPS types was performed by multiplex PCR. This PCR method using different four specific primer pairs was carried out to differentiate and compare with other bacterial strain differential methods. For instance, results of this PCR method was used to classify strains, which was important in assessment of host range analysis (Table 9).

Materials:

All materials listed under 3.3.3 except the following changes in primers and ladders used, in this CWPS typing: 100 bp DNA Ladder and four types of *L. lactis* specific primers were used (Table 3).

Table 3 . Four primer pairs used during Multiplex PCR

Primer	**Sequence (5′–3′)**	**Product size (bp)**
IL-KFfw	GATTCAGTTGCACGGCCG	
IL-KFrv	AGTAAGGGGGCGGATTGTG	183
MG-SKfw	AAAGCTCATCTTTCCCCTGTTGT	
MG-SKrv	GCACCATAGTCTGGAATAAGACC	686
UC-CVfw	GTGCCTATGCTCCGTTAGTC	
UC-CVrv	CGAGGGCCAATCTCTTTACC	442
CONfw (control)	GTACACTATGTTTATAACAATCATCCAG	
CONrv	GCAAACCAGATTCAAAGTCAGTATG	891

Source: Mahony *et al*. (2013b).

Procedures:

In this method 91 strains were differentiated by using four primer pairs as described by Mahony *et al*. (2013b). Briefly three primer pairs including IL/KF, MG/SK and UC/CV were applied in a single PCR reaction. A control was also included, where primers based on the conserved *rmlB* gene were used to generate a product of 891 bp, so that to confirm the PCR was working in all samples. For PCR amplification, the DNA template and master mix preparation and reaction volume was the same as mentioned for $(GTG)_5$-PCR, except 3 primer pairs ad control primers were used in this PCR amplification.

The three primer pairs were composed of 1μL of each the following components IL/KF-F, IL/KF-R, MG/SK-F, MG/SK-R, UC/CV-F, UC/CV-R, and control both forward and reverse primers. The PCR amplifications of the three primer pairs were performed in a Thermal Cycler, with an initial denaturation at 95°C for 1 min, 30 cycles of denaturation at 95°C foe 20 sec, annealing at 55°C for 20 sec, elongation at 72°C for 1 min, and final extension at 72°C for 5 min. The PCR products were visualized using a 100 bp DNA ladder and 1.5 % Agarose gel at 90 Volt for about 30-40 minutes.

4. STATISTICAL ANALYSIS

The statistical analysis of our results was performed with the application of different software programs and online statistical software and basic statistics.

i. The phenotypic data of both bacterial strains and phages were analyzed by constructing a dendrogram by complete linkage Euclidean distance analysis using Minitab software.

ii. The genomic data $(GTG)_5$-PCR fingerprints were analysed by a computer Gel compare II, using Pearson's correlation coefficients with unweighted pair group method with arithmetic averages clustering algorithm (UPGMA) analysis method.

iii. Online statistical software was used to analyse chai square test, to asses relationship between CWPS genes type of strains and their phage susceptibility to phage groups (http://www.physics.csbsju.edu/stats/contingency_NROW_NCOLUMN_form.html).

iv. Some basic statistics like percentage analysis was also used when necessary.

5. RESULTS

5.1 Detection of phages in bulk starters and whey samples

To detect the presence of infectious phages and determine susceptibility of bacterial isolates, plaque assay in 10 fold dilution series was performed using three bulk starters and three whey samples. As indicated below (Fig. 1), the first two whey samples (left side), followed by three bulk starters collected, on July 17, 18 and 19/2013 (BS17/7, BS18/7 and BS19/7), and the sixth one is another whey sample. Whey samples are indicated as wheyx, wheyy and wheyz, this is only to show that they were different samples. Infection was considered positive when plaque was seen in these tests. The same tests applied to all the 96 strains, and plaque producing phages were obtained from 40 indicator strains. The highest phage concentration was mostly observed in BS samples (Fig. 1. C11/#835, D12/#848).

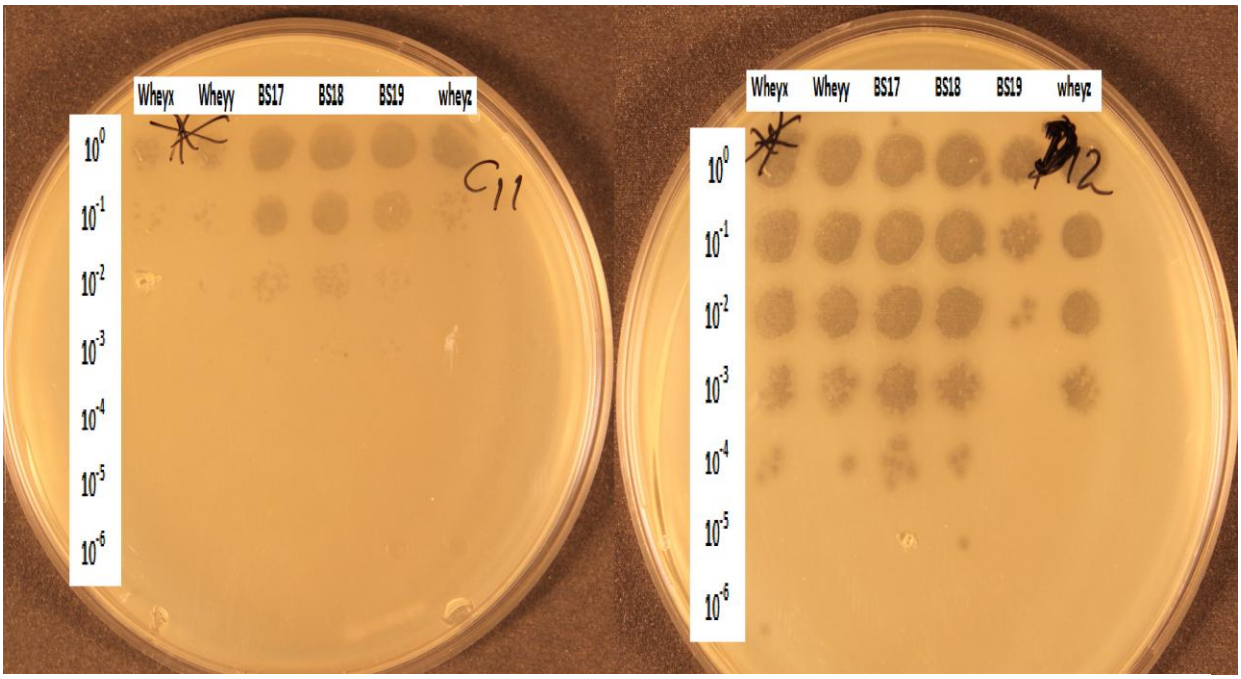

Fig. 1**.** Schemes in plaque assay using bulk starter and whey samples. Before inoculation, bulk starter and whey samples were diluted at 10 fold dilution series, the highest phage concentration was shown at zero or lower dilutions.

Following the isolation of the forty plaque producing phages, they were propagated on their respective hosts, supernatant of each strain was collected, filtered and maintained at 4ºC (Table 4).

Table 4. List of 40 phages isolated from their respective bacterial strains

Φ811	Φ835	Φ839	Φ884
Φ813	Φ827	Φ840	Φ874
Φ822	Φ828	Φ861	Φ875
Φ815	Φ830	Φ870	Φ876
Φ816	Φ831	Φ862	Φ877
Φ817	Φ832	Φ863	Φ878
Φ818	Φ837	Φ864	Φ879
Φ819	Φ847	Φ867	Φ880
Φ820	Φ848	Φ868	Φ888
Φ834	Φ838	Φ873	Φ889

5.2 Phage typing of strains with non-purified phages

Phage typing of the non-plaquing strains was studied using non-purified phages. Spots from the 56 strains were taken from the agar surface previously stamped with the samples, and added in 10ml of LM17 on their respective hosts. Growth of most strains was inhibited and 40 supernatant from the sensitive strains were filtered and spotted to all the 56 initially non plaque forming strains. The use of non-purified phages showed the sensitivity of 51 previously untargeted strains. This high number of bacterial sensitivity can be supported by the growth kinetics study; where the growth of 88% (80/91) strains was inhibited by the bulk starters (Table,7, Fig. 6).

Among the different LAB, only two strains (844 and 855) showed an overlap in sensitivity to the non purified phages (Fig. 2). Similarly, PCR methods showed both have type B CWPS (Table 8). $(GTG)_5$-PCR also grouped them under the same major cluster but different subgroups (Fig.9B). The results of these two unique strains showed same plaque results from their non-purified phages; moreover, growth kinetics study showed both LAB strains were sensitive to phage infection. However, both strains were not targeted by the phages in the bulk starter and phages purified from plaques.

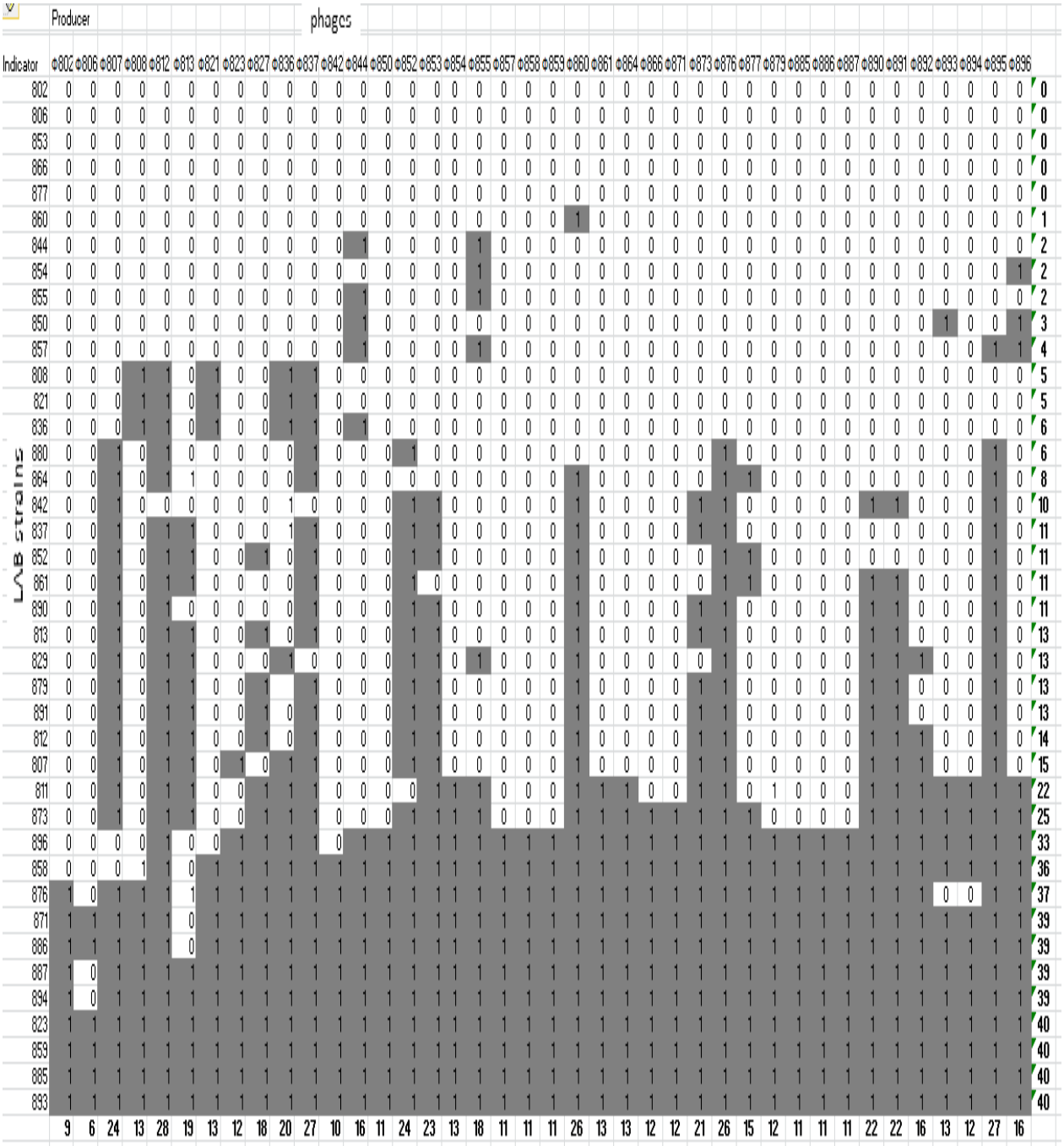

Fig. 2. Host ranges of 40 non-purified phages. Grey regions represent lysis zones and white regions indicate no lysis.

This figure 3, indicates the host range of non-purified phages that were taken from the surface agar non-plaqueing strains. In this method most phages had wide host ranges, particularly; Φ806, Φ812, Φ837, Φ852, Φ853, Φ860, Φ876 and Φ895 were phages which attacked high number of strains. High sensitivity was mainly observed in strains 893, 885, 59, 823, 894, 887, 886, 871 and 876. Among the initially untargeted strains, four strains (B11/#823, E11/#859, H9/#889 and H1/#885) were highly sensitive to all tested phages (Fig. 3). Others were attacked with smaller number of phages (Fig. 2).

Broad host spectrum activity was observed in most of the phages. After several plaque assay tests, except five strains (#802, #806, #853, #866 and #877), 51 strains were found susceptible to different phages (Fig. 2 and 3).

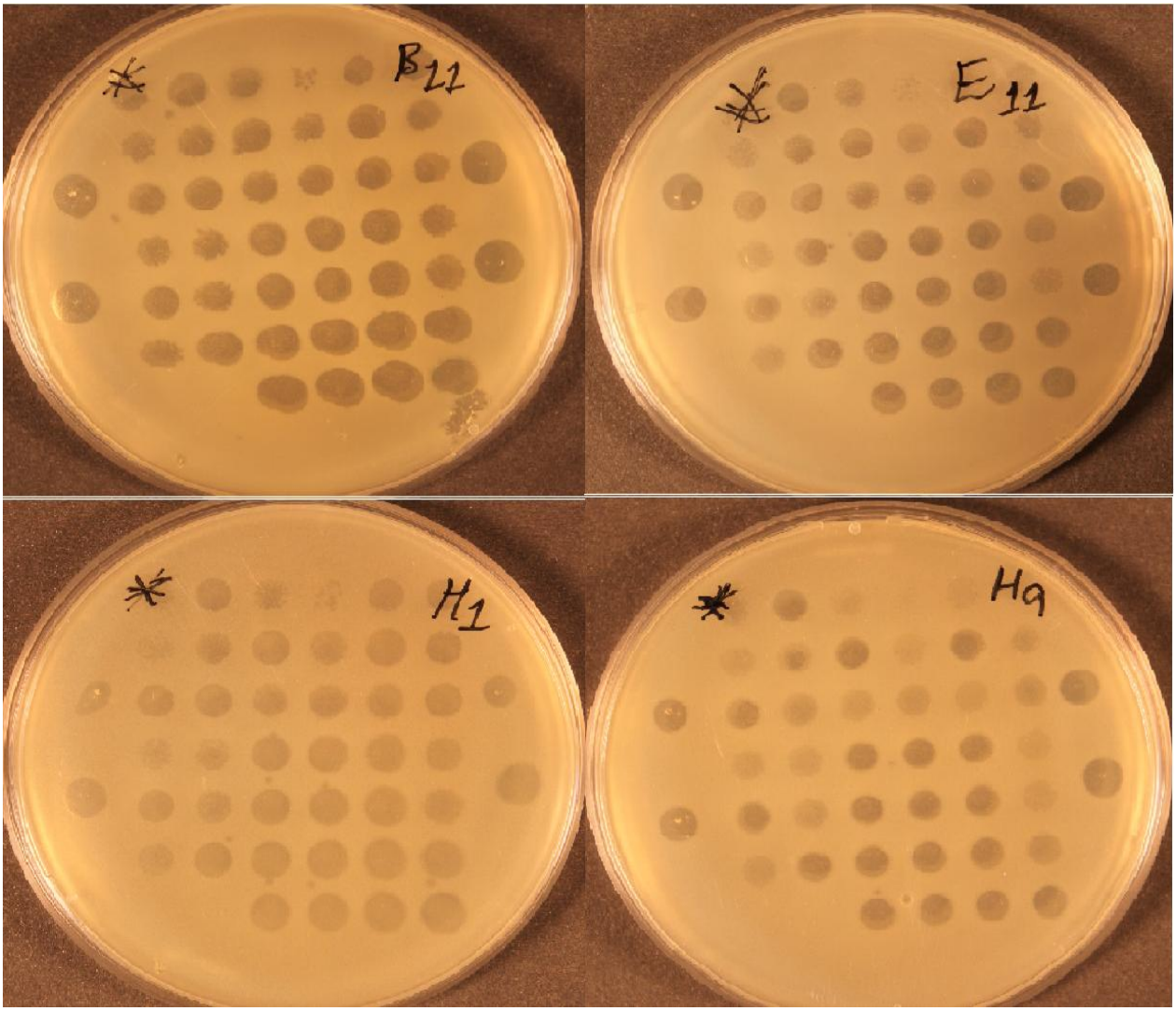

Fig. 3. Broad host ranges of phages and high sensitivity strains. Since the same inoculum of phages was used to infect all plates, these four strains were sensitive to all the non-purified phages, at the same time phages also showed broad host spectrum.

5.3 Phage typing of the 96 strains with phages purified from plaques

To examine whether the phages were specific or broad host spectrum, all the 96 strains were exposed to the same phage inoculum obtained from the pure plaque filtrates. This was performed for each forty different phages, and when bacterial growth was slow infectivity was examined within 1, 2, and 3 days and results were recorded based on the plaque assay. The scheme of the phage used to infect all the strains are indicated (Table 5), approximately 5µL per phage suspension per spot was used to infect each strain.

Table 5. Scheme on 96 well plate used during phage typing of bacterial strains

	1	2	3	4	5	6
A	Φ811	Φ813	Φ815	Φ816	Φ817	Φ818
B	Φ819	Φ820	Φ822	Φ827	Φ828	Φ830
C	Φ831	Φ832	Φ834	Φ835	Φ837	Φ838
D	Φ839	Φ840	Φ847	Φ848	Φ861	Φ862
E	Φ863	Φ864	Φ867	Φ868	Φ870	Φ873
F	Φ874	Φ875	Φ876	Φ877	Φ878	Φ879
G	Φ880	Φ884	Φ888	Φ889		

In this test 16 strains were found insensitive to those 40 phages isolated from pure plaques, those untargeted strains were: #802, #804, #808, #809, #814, #815, #824, #36, #841, #844, #850, #853, #855, #866, #866 and #872. Unlike the previous test, where high susceptibility of strains to multiple phage infections observed (Fig. 3), only one bacterial strain (F10/#870) was susceptible to all the isolated phages (Fig. 4).

After phage typing of all bacterial strains, the phage host range and the bacterial susceptibility to specific phages were analyzed and dendrograms were constructed for both the bacterial strains and phages using a Minitab Software. The susceptibility to phage infection was different from strain to strain. The phage host range and distribution of phage sensitivity of bacterial isolates are indicated in following figure (Fig. 4).

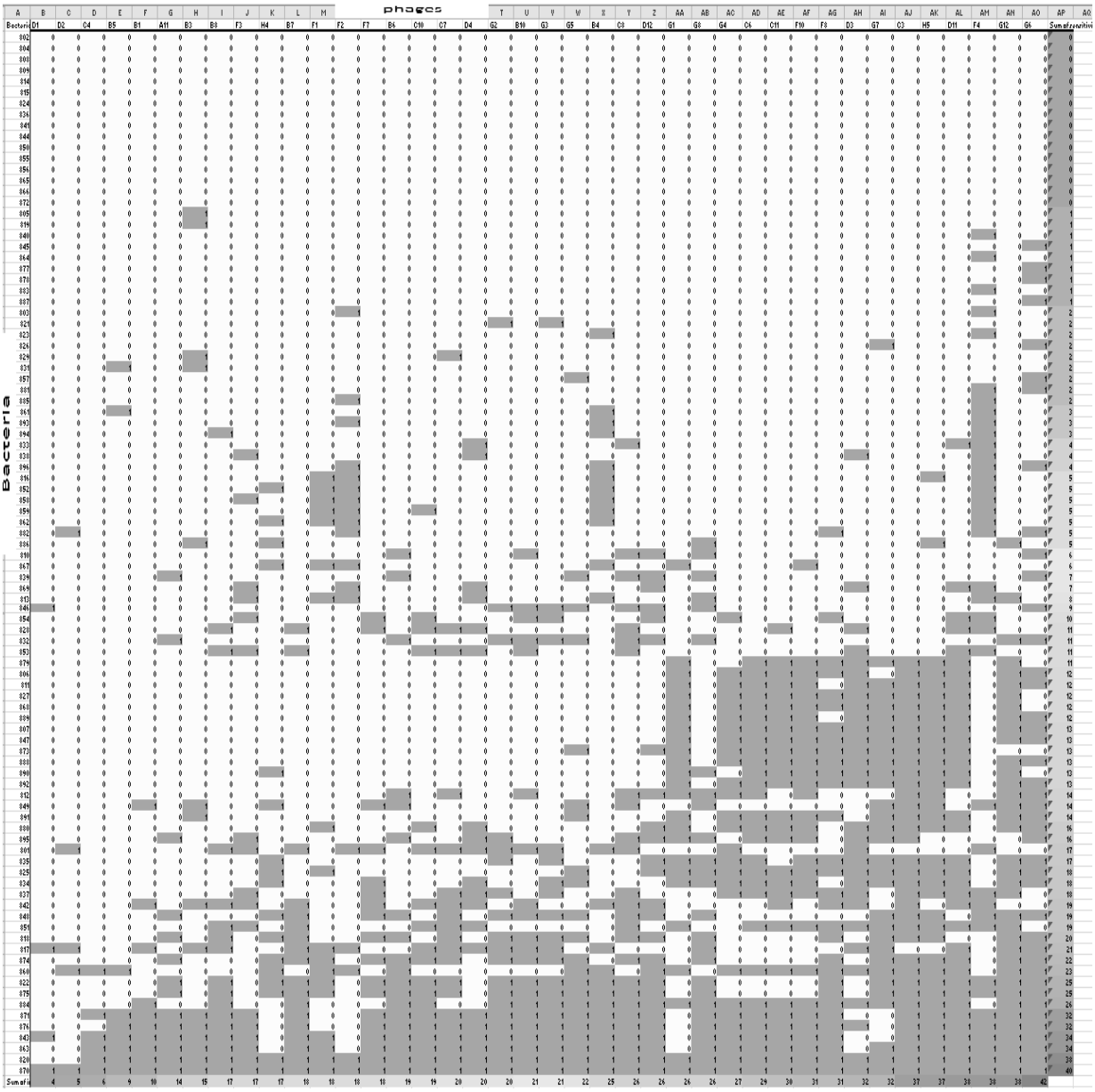

Fig. 4. Host ranges of the 40 purified phage isolates. Grey areas indicate lysis of the susceptible hosts and white fields indicate that no inhibition was detected.

5.4 Differentiation of phages

All phages assessed in this study had a relatively broad host range varying from the least four (Φ837) to most forty two (Φ878) different hosts. Based on the complete linkage Euclidian distance analysis, the forty phage isolates were used to construct a dendrogram which has two major clusters, and in one of these there are four sub clusters. Each cluster contains phages with close phenotypic relationship. This cluster analysis indicates their relationship on how close/far they are to target similar hosts. Thus, based on their host range, the forty phages were classified in to four groups. The number of similar phages (Φs) per group are indicated in brackets, Group, 1 (6 Φs), 2(10 Φs), 3(12 Φs) and 4 (12Φs).

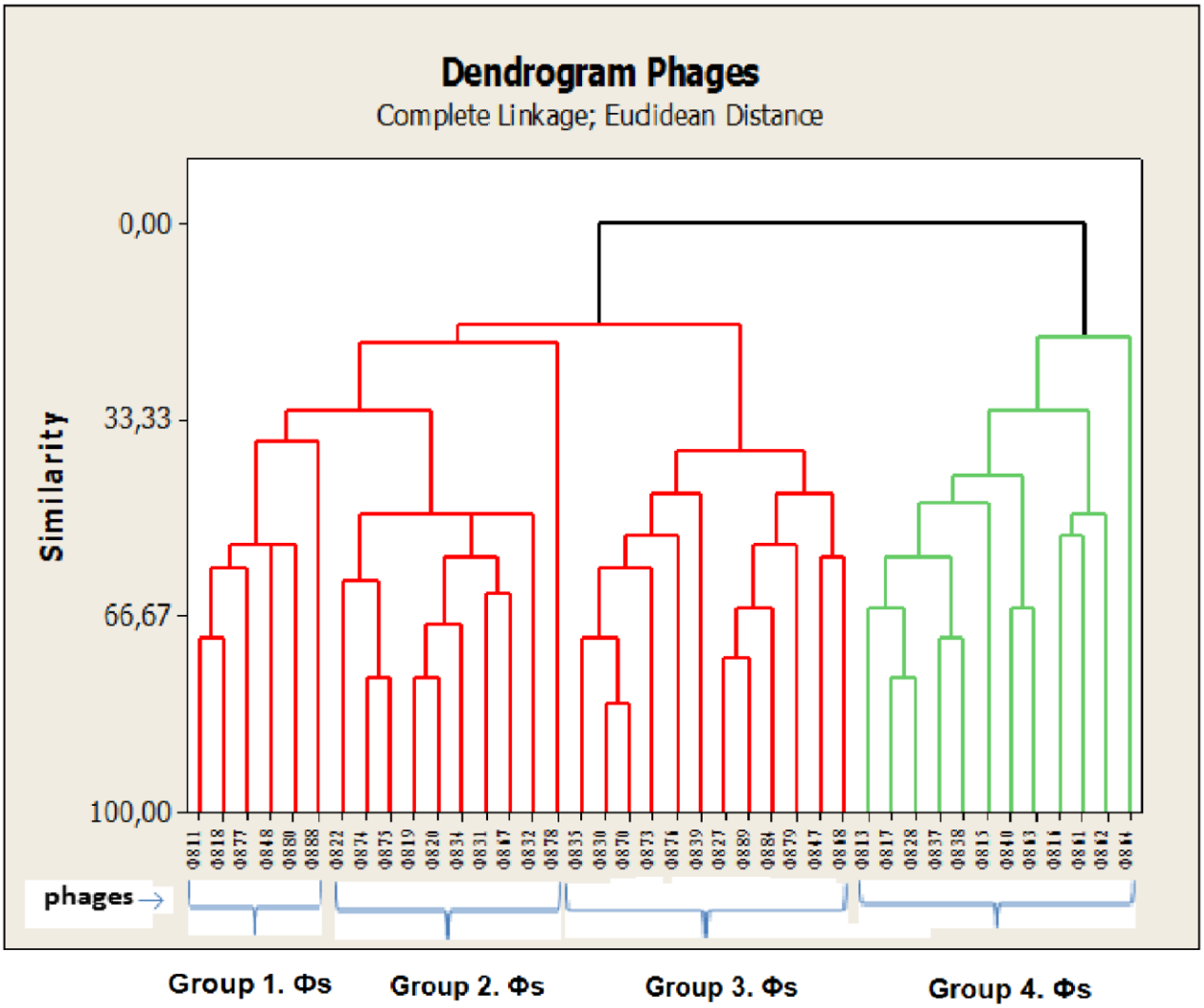

Fig. 5. Dendrogram of 40 purified phage isolates (Φs). The four groups of phages were arranged in order of increasing their host spectrum.

5.5 Fermentation inhibition test

The inhibition of lactose fermentation by dairy starter culture may be caused by different factors, but phage infection is the known cause of fermentation failure. To determine the impact the isolated phages on lactose fermentation, starter culture S, was grown in reconstituted 10% skim milk. Each test sample had one blank, two control and test tubes for each fermentation test. Test tubes received 3% of actively growing culture S and 0.25% of a mixture of phages isolates. The control tube received culture S only. Cultures were incubated at 30ºC for 4 hrs and acidity was determined by pH measurement using a pH meter. Our results showed reduction in acid production and one test sample treated with 79 phages was severely attacked.

From this experiment, two of the tests samples, namely: test sample 3 which was treated with a mixture of 6 broad spectrum phages (Mix 1) and test sample 4 which was received a mixture of 21 mainly narrow-spectrum phages (Mix 2) shown under Appendix 1, caused a slight reduction in acid production. Both test sample 3 and 4 respectively showed 0.05 and 0.06 less pH drop during fermentation activity of the starter cultures respectively. On the other hand, test sample 5, which was treated with mixture of all the 40 isolated phages (Mix 3) showed 0.22 less pH drop than the control. Similarly, test sample 6, which was treated with another 39 phages isolated on strains of culture S origin (Mix 4, from Cyril Frantzen) showed 0.21 less pH drop than the control. Finally, one treatment group (test sample 7), caused the highest inhibition of bacterial activities, shown by reduction of acid production. This test culture was treated with a mixture of 40 and 39 (Mix 3 and Mix 4) phage isolates (Mix 5), resulting in 0.36 reduction in pH than the control.

Table 6. Test score of fermentation inhibition tests

Test sample	Phage mixtures	pH1	pH2	Average pH	Activity (pH change)
1	-	6,65		6,65	
2	-	5,58	5,73	5,66	1.00
3	6	5,7	5.7	5,70	0.95
4	21	5,71	5.71	5,71	0.94
5	40	5,88	5,86	5,87	0.78
6	39	5,79	5,94	5,87	0.79
7	79	6,01	6,01	6,01	0.64

5.6 Differentiation of strains by phage typing and PCR methods

5.6.1 Growth inhibition in bacterial isolates

Phage sensitivity of all the strains was studied during their growth kinetics. For the determination of growth inhibition test, isolates were grown in LM17 broth supplemented with 5mM $CaCl_2$ solution. The inhibition effect of phages in bulk starter was examined during 16 hrs incubation time in a microtiter plate reader.

Most of the strains that were treated with bulk starter samples were attacked as shown by their growth inhibition. In the control group all strains except 4 (802, 822, 847 and 884) grew in LM17 Medium; whereas, growth in most bulk starter treated groups was affected by the addition of BS. Moreover, among the 16 non plaqueing strains detected by the plaque assay

method, 11 of these strains (808, 809, 815, 824, 836, 841,844, 850, 855, 865 and 866) were inhibited in this growth kinetics study (Fig. 6). The growth of four strains (804, 814, 856 and 872) was not affected by both plaque assay and during growth kinetics.

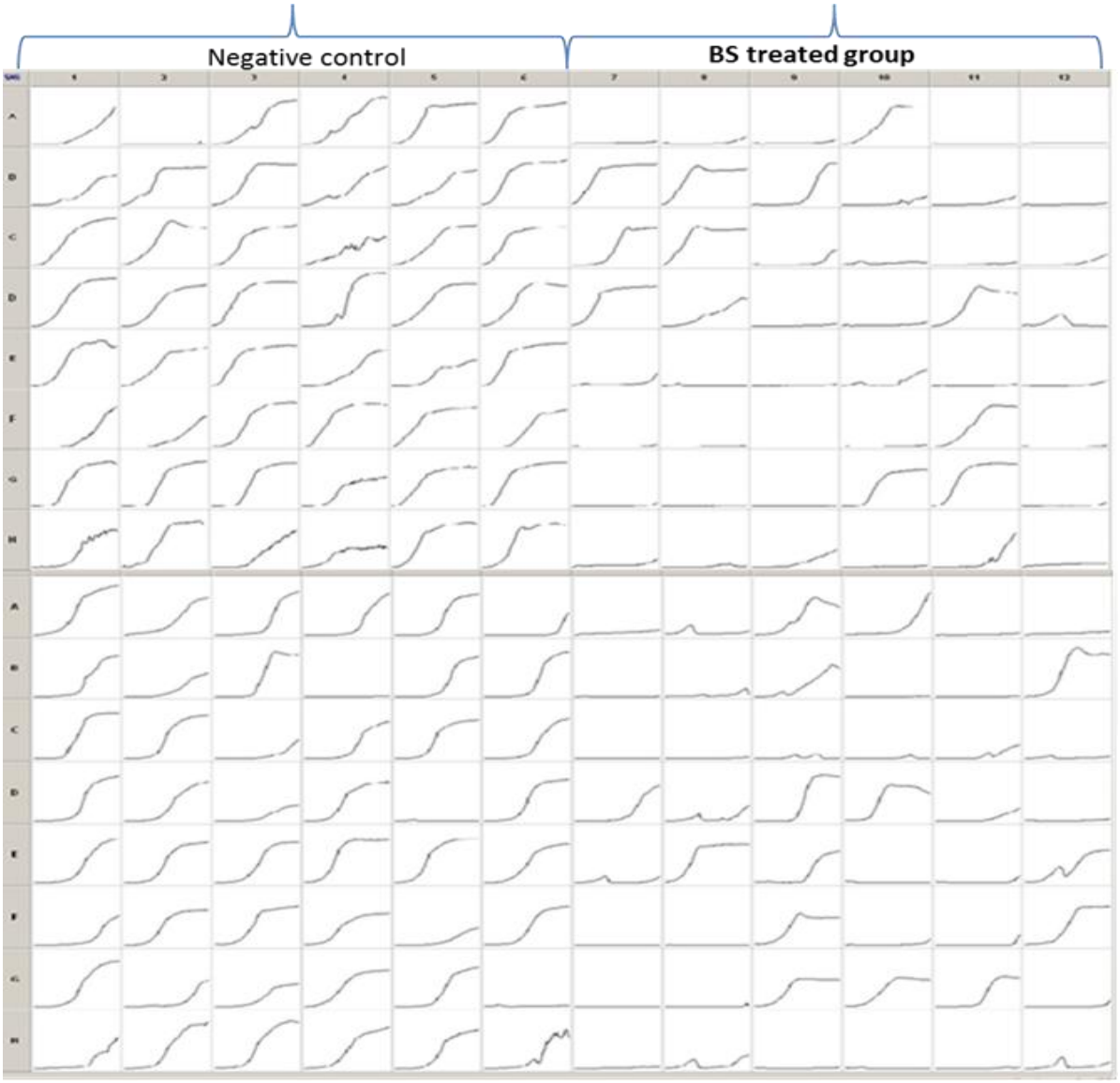

Fig. 6. Phage attack of bacterial strains studied by growth kinetics. The growth inhibition of strains by the addition of bulk starter (BS) sampled on July 19/2013 from cheese plant in Nærbø, indicated that 81 strains were affected by bulk starter treatment; where 39 of them were totally inhibited (no growth = X), and 42 showed various level of sensitivities (shown under Table 6).

In this study, out of the 96 bacterial isolates, four (802, 822, 847 and 884) were not grown during this test. Thus only 92 strains were considered in the growth inhibition test. From the treatment group, unexpectedly one strain (D9/845) gave a strange result, while the growth of 11 strains was not inhibited by BS treatment. About 88% (80/91) strains were attacked by the addition of BS and their growth was either partially or totally inhibited (Fig. 6).

Table 7. Summary of phage attack study by growth kinetics

	Control	BS treated group		Control	BS treated group		Control	BS treated group
LAB strains	Growth (+/-)	Growth (+,-, X)	LAB strains	Growth (+/-)	Growth (+, -, X)	LAB strains	Growth (+/-)	Growth (+, -, X)
801	+	X	833	+	-	865	+	-
802	-		834	+	-	866	+	X
803	+	-	835	+	-	867	+	X
804	+	+	836	+	-	868	+	X
805	+	X	837	+	+	869	+	-
806	+	X	838	+	-	870	+	X
807	+	-	839	+	X	871	+	-
808	+	-	840	+	X	872	+	+
809	+	-	841	+	-	873	+	X
810	+	+	842	+	-	874	+	X
811	+	X	843	+	-	875	+	X
812	+	X	844	+	-	876	+	+
813	+	+	845*	+		877	+	+
814	+	+	846	+	-	878	+	X
815	+	-	847	-		879	+	X
816	+	-	848	+	X	880	+	X
817	+	-	849	+	-	881	+	+
818	+	X	850	+	X	882	+	-
819	+	X	851	+	X	883	+	-
820	+	-	852	+	-	884	-	
821	+	-	853	+	X	885	+	-
822	-		854	+	-	886	+	-
823	+	X	855	+	-	887	+	-
824	+	-	856	+	+	888	+	X
825	+	-	857	+	-	889	+	-
826	+	+	858	+	X	890	+	X
827	+	-	859	+	X	891	+	X
828	+	-	860	+	-	892	+	-
829	+	X	861	+	X	893	+	X
830	+	-	862	+	X	894	+	X
831	+	X	863	+	X	995	+	X
832	+	X	864	+	X	896	+	-

BS was tested against all the 96 strains and growth (+) and growth inhibition (-) and no growth (X)

* Means slowly growing strain in the control, but faster growth in the BS treated culture, that is not expected but it may be caused by some technical errors.

5.6.2 Phage typing of strains

A collection of 96 LAB strains isolated from cheese production process (1 hour after starter S was added to the milk) were analyzed with both phage typing and PCR methods to assess their susceptibility to phage infection and relatedness. Based on the results of these tests, differentiation of bacterial strains was done. During a plaque assay method, results showed that most strains were highly susceptible while others were resistance to phage infection. Thus phage susceptibility of strains was highly variable. Based on the dendrogram developed from the phage typing (Fig. 7), phenotypic relatedness between strains was differentiated in to 6 groups. This cluster analysis was used to evaluate the association between phage sensitivity of the strains and the nature of their CWPS types. However, one of the clusters which contain 16 strains was not sensitive to the isolated phages, as a result we excluded from this assessment study. Accordingly, strains of group 2 to 6 were used to test relationship between the two factors (Table 11).

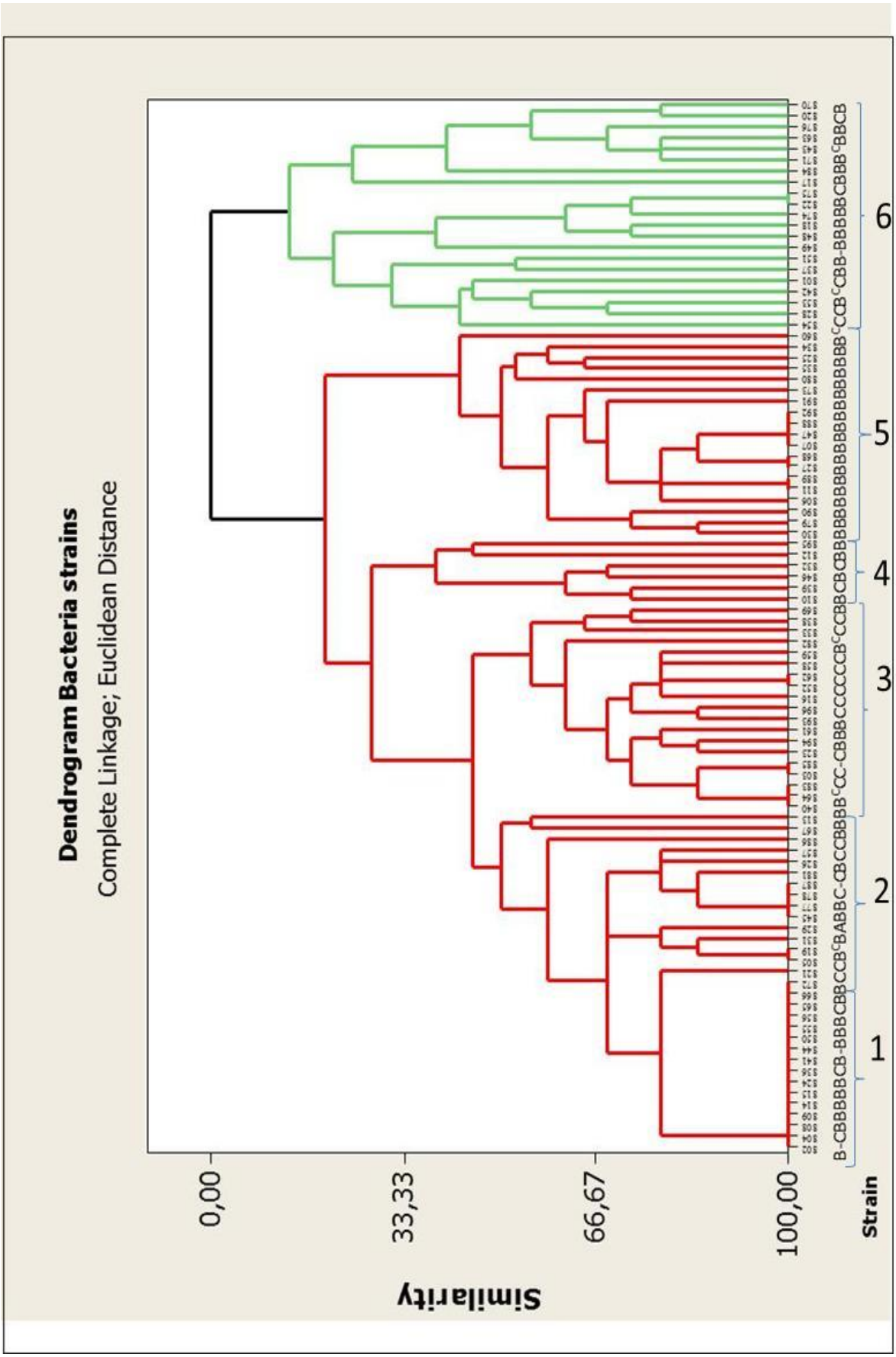

Fig. 7. Differentiation of bacterial strains based on their susceptibility to the 40 purified phage isolates. B^C means strains that were detected to have both CWPS genes B and C. CWPS negative indicated by dash (-).

5.6.3 Differentiation of bacterial isolates by $(GTG)_5$-PCR fingerprint analysis

The $(GTG)_5$-PCR fingerprinting profiles of the 96 strains used in this study is shown (Fig. 9). In this test all strains gave signals and differentiated by different number of bands, but most strains showed similar band patterns.

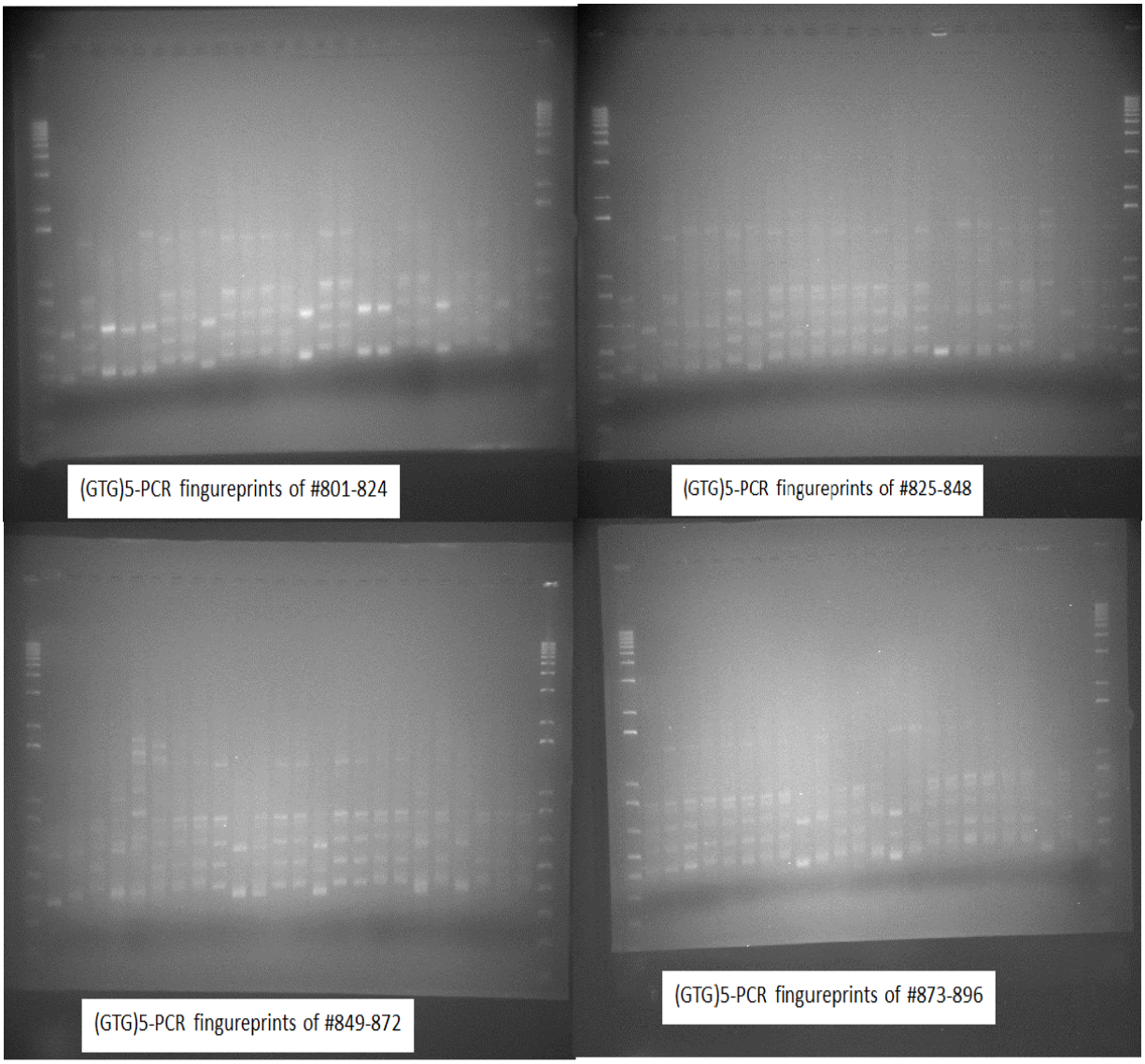

Fig. 8. Differentiation of strains by $(GTG)_5$-PCR banding patterns. Lanes of strains are numbered from left to right.

Based on Pearson's correlation coefficients with UPGMA analysis method, fingerprinting results revealed two main distinct clusters, the first main cluster (group 1), visually different fingerprints were shown revealing about 74% similarity between those strains. While the second cluster included seven highly similar seven subgroups which indicated as group 2 to 8 (Fig. 9B), this subgroups revealed similar results with similarity level of > 83 % (Fig. 9B).

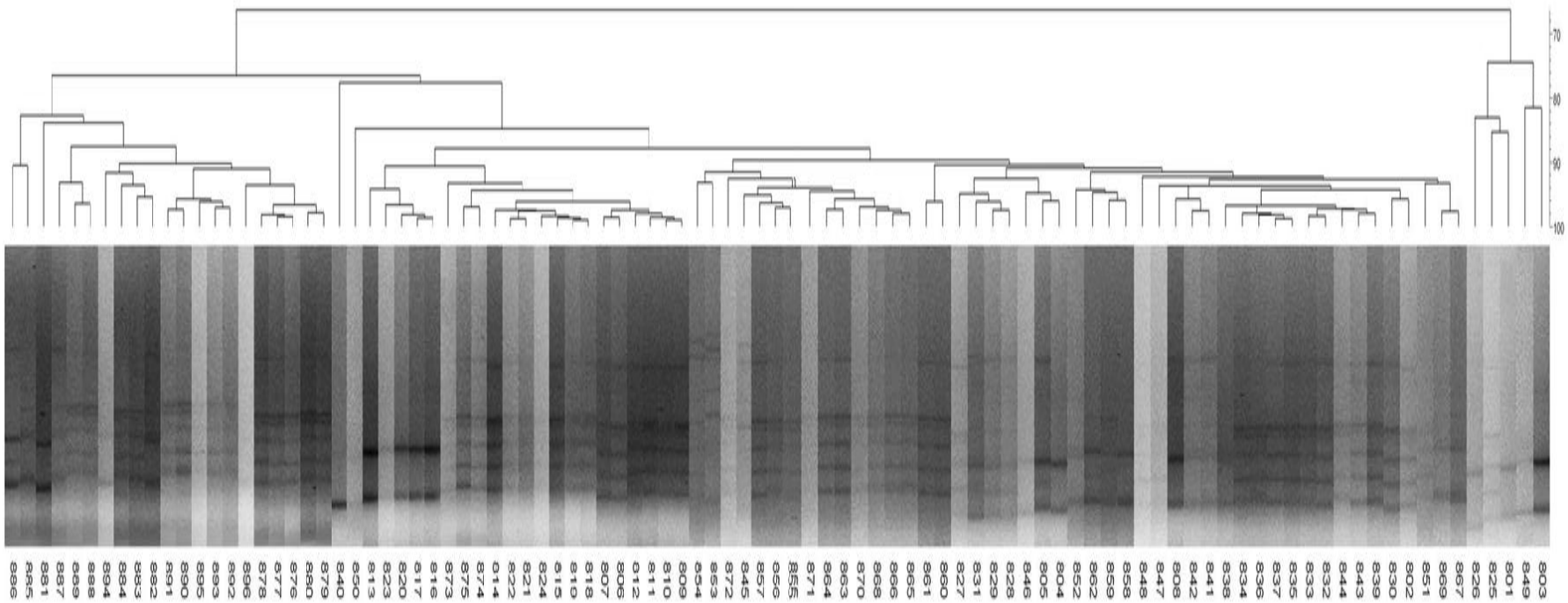

Fig. 9A. Dendrogram based on cluster analysis of $(GTG)_5$-PCR fingerprinting profiles revealed by 96 LAB strains. The dendrogram was generated after cluster analysis of the digitized (GTG)5-PCR fingerprints with Pearson's correlation coefficients using UPGMA clustering method.

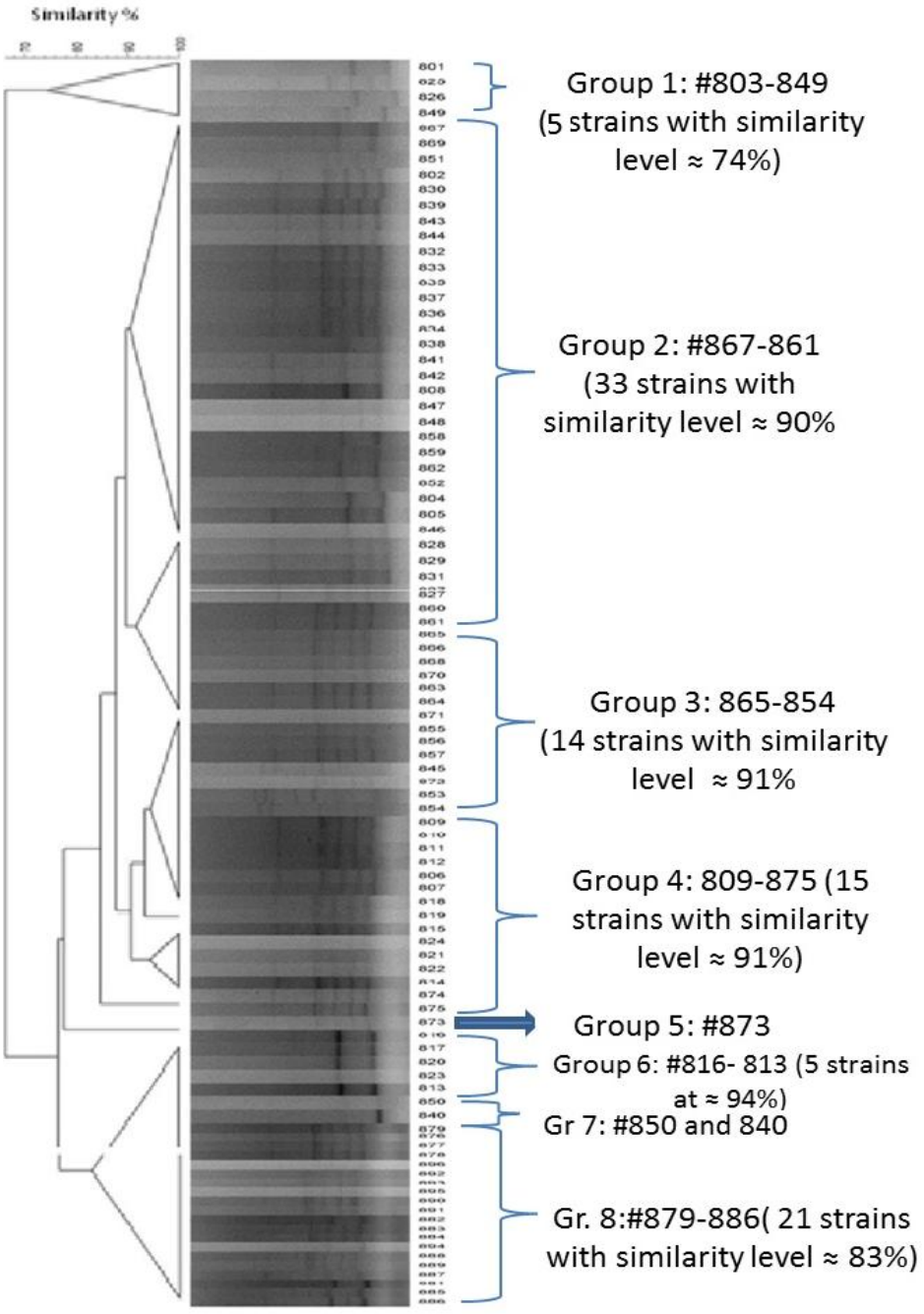

Fig. 9B. Dendrogram obtained by cluster analysis of $(GTG)_5$-PCR fingerprint patterns demonstrating the relatedness between LAB strains. Strain members of each group are indicated with their similarity level, however, three strains (#840, #850 and #873) were observed with visible bands but the cluster analysis didn't include them in the grouping, so they are considered as separate groups.

This dendrogram is developed from $(GTG)_5$-PCR fingerprinting profile indicated in Fig. 9A, when closely similar bands where merged for genotypic grouping, the fingerprinting method separated the analyzed strains in to six groups. The 8 groups are: group 1 (G1), includes 5 strains (803, 849, 801, 825 and 826) with similarity level 74%. Group 2, includes 33 strains (867, 869, 851, 802, 830, 839, 843, 844, 832, 833, 835, 837, 836, 834, 838, 841, 842, 808, 847, 848, 858, 859, 862, 852, 804, 805, 846, 828, 829, 831, 827, 860, and 861) with 90% group similarity. Group 3, contains 14 strains with 91% similarity and those strains were 865, 866,868, 870, 863, 864, 871, 855, 856, 857, 845, 872, 853 and 854. Group 4 include 15 strains (809, 810, 811, 812, 806, 807, 818, 819, 815, 824, 821, 822, 814, 874 and 875) this group has relatively the highest percentage of similarity (94%). Group 5 contain one strain only, which was not included in the merging process, Group 6 contain 5 strains (816, 817, 820, 823 and 813) like group 4 strains had 94% similarity. Group 7 contain 2 strains (840 and 850), like group 5 these strains were not included in the grouping. Finally group 8 includes 21 strains which include: 879, 880, 876, 877, 878, 896, 892, 893,894, 895, 890, 891, 882, 883, 884, 888, 889, 887, 881, 885 and 886 in group similarity level between the strains was revealed to be 83%. Based on the $(GTG)_5$-PCR fingerprint results, most strains particularly in the second major cluster which included group (G) 2 to 8 were closely related with a similarity level > 83%.

5.6.4 CWPS genes typing of strains by multiplex-PCR

The diversity of the strains was further evaluated by multiplex-PCR using three primer pairs amplifying the genes encoding for different groups of CWPS. In most of the strains, this method resulted in detection of CWPS genes shown by visible bands. Based on similarity in CWPS encoding genes it was possible to divide strains into five groups (Table 8).

Our results showed about 95% genes of the tested strains were targeted by the primers used in this study. Moreover, about 60% and 30% genes of the strains were specifically differentiated as CWPS type B and C respectively.

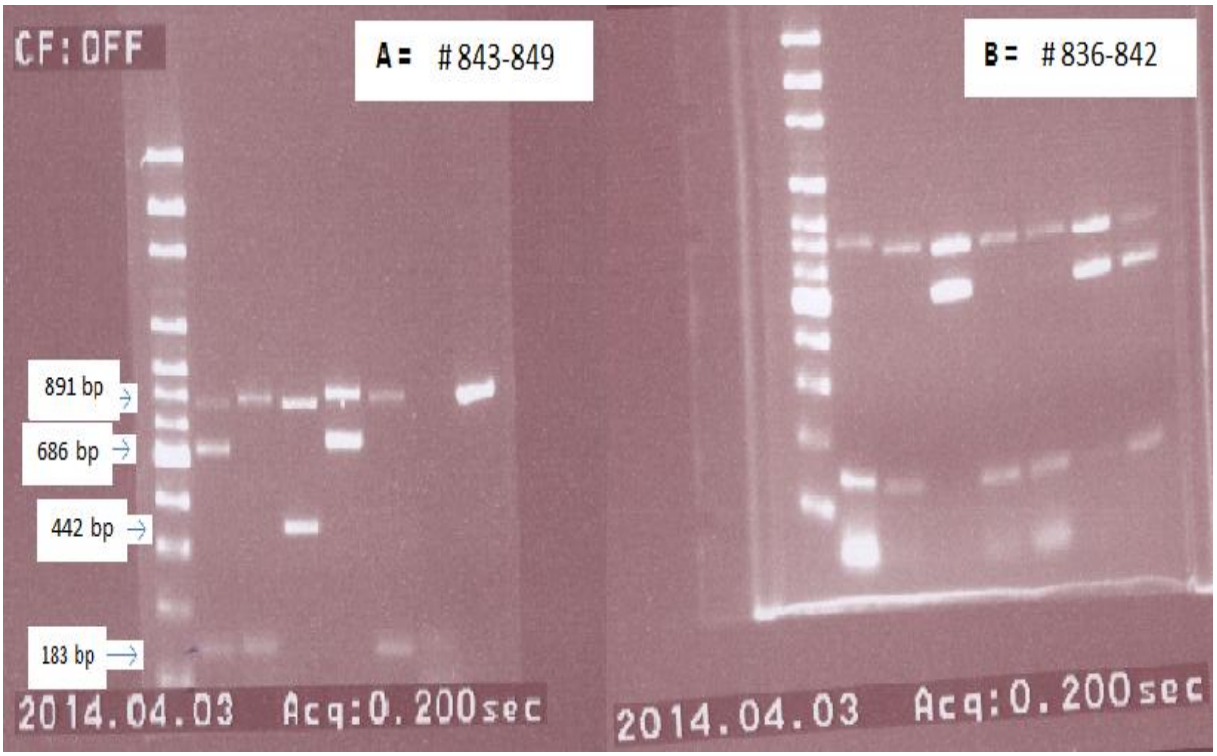

Fig. 10. Products of PCR assays showing different target CWPS genes. The differentiation scheme for strains in these representatives results show seven strains detected to have genes encoding three CWPS groups; B(183 bp), A(442 bp), C(686) and controls primer (891 bp) showing the validity of the PCR reaction. The control for the multiplex PCR was based on the *rmlB* gene, which is conserved in each of the strains (Mahony *et al*., 2013b). Lanes of strains are numbered from left to right. Gel A (left side), detected three different CWPS genes encoding CWPS type A, B and C.

Based on the overall analysis by multiplex PCR, the total findings are summarized below (Table 8).

Table 8. Comparison of strains based on genomic regions encoding their CWPS.

B			C		A	B and C	Non- CWPS
#802	#835	#871	#801	#866	#845	#831	#804
#806	#836	#872	#803	#867		#833	#823
#807	#837	#873	#805	#869		#842	#849
#809	#839	#874	#808	#882		#843	#850
#810	#840	#875	#812	#885		#854	#881
#811	#844	#876	#816	#886		#883	
#813	#847	#877	#817	#887			
#814	#848	#878	#819	#894			
#815	#851	#879	#820	#896			
#818	#855	#880	#826				
#821	#856	#884	#828				
#822	#857	#888	#838				
#824	#860	#889	#841				
#825	#861	#890	#846				
#827	#863	#891	#852				
#829	#864	#892	#853				
#830	#865	#893	#858				
#832	#868	#895	#859				
#834	#870		#862				
Total	**56**		**28**		**1**	**6**	**5**

Differentiation of strains by multiplex PCR.

5.6.5 Comparison of methods used for differentiation of strains

Various molecular techniques have been developed to characterize and differentiate bacterial populations. Based on the variation between the methods used in this study, we compared methods that are commonly used for differentiation of bacterial strains (Table 9), and results indicated that high resolution was found in phage typing which may help to differentiate *L. lactis* up to various strain levels. On the other hand, more homogeneous results were obtained from both PCR methods, which can be used to differentiate species and subspecies and may be strains (Gevers *et al.,* 2001), but their resolution is lower than the phage typing method. Another molecular method which has been widely used for the identification and classification of LAB is based on the application of rRNA, because the various degrees of

sequence conservation within the different regions of 16S rRNA allows the assay to be used to the desired specificity (Erlandson and Batt, 1997).

Table 9. Comparative analysis of LAB obtained by the different methods

Strain #	16S rRNA PCR	CWPS typing	$(GTG)_5$-PCR	Plaque assay	Strain #	16S rRNA PCR	CWPS typing	$(GTG)_5$-PCR	Plaque assay	Strain #	16S rRNA PCR	CWPS typing	$(GTG)_5$-PCR	Plaque assay
801	1	2	1	6	833	1	3	2	3	865	1	1	3	1
802	1	1	2	1	834	1	1	2	5	866	1	2	3	1
803	1	2	1	3	835	1	1	2	5	867	1	2	2	2
804	1	4	2	1	836	1	1	2	1	868	1	1	3	5
805	1	2	2	2	837	1	1	2	6	869	1	2	2	3
806	1	1	4	5	838	1	2	2	3	870	1	1	3	6
807	1	1	4	5	839	1	1	2	4	871	1	1	3	6
808	1	2	2	1	840	1	1	7	3	872	1	1	3	1
809	1	1	4	1	841	1	2	2	1	873	1	1	8	5
810	1	1	4	4	842	1	3	2	6	874	1	1	4	6
811	1	1	4	5	843	1	3	2	6	875	1	1	4	6
812	1	2	4	4	844	1	1	2	1	876	1	1	6	6
813	1	1	5	2	845	1	5	3	2	877	1	1	6	2
814	1	1	4	1	846	1	2	2	4	878	1	1	6	2
815	1	1	4	1	847	1	1	2	5	879	1	1	6	5
816	1	2	5	3	848	1	1	2	6	880	1	1	6	5
817	1	2	5	6	849	1	4	1	6	881	1	4	6	2
818	1	1	4	6	850	1	4	7	1	882	1	2	6	3
819	1	2	4	2	851	1	1	2	6	883	1	3	6	3
820	1	2	5	6	852	1	2	2	3	884	1	1	6	6
821	1	1	4	2	853	1	2	3	6	885	1	2	6	3
822	1	1	4	6	854	1	3	3	6	886	1	2	6	2
823	1	4	5	3	855	1	1	**3**	1	887	1	2	6	2
824	1	1	4	1	856	1	1	**3**	1	888	1	1	6	5
825	1	1	1	5	857	1	1	3	2	889	1	1	6	5
826	1	2	1	2	858	1	2	2	3	890	1	1	6	5
827	1	1	2	5	859	1	2	2	3	891	1	1	6	5
828	1	2	2	6	860	1	1	2	5	892	1	1	6	5
829	1	1	2	2	861	1	1	2	3	893	1	1	6	3
830	1	1	2	5	862	1	2	2	3	894	1	2	6	3
831	1	3	2	2	863	1	1	3	6	995	1	1	6	4
832	1	1	2	4	864	1	1	3	3	896	1	2	6	3

CWPS type 1 means = B, 2= C, 3 = B and C, 4 = not detected and 5 = A

Though 16S rRNA is not used in this study, it is included for comparison purpose, because this method can differentiate *L. lactis* at the lowest resolution, though sequencing can differentiate subspecies of *L. lactis* (Holmfeldt *et al*., 2007). Thus, when differentiation below the subspecies level is required, the use of 16S rRNA genes are no longer helpful since the nucleotide sequences of these genes are too conserved to allow the design of strain-specific probes (Koehler *et al.,* 1991).

5.7 Association between CWPS encoding genes and phage sensitivity of strains

Phage infection requires the recognition of bacterial cell surfaces by their RBPs. Those RBPs are well-known to bind to carbohydrate cell surface receptors and may determine the specificity and host range of phages (Shibata *et al*., 2009). Moreover, genome analysis on strains of *L. lactis* species suggests that there are three different CWPS groups synthesized and are believed to have a role as a phage receptor (Mahoney *et al*., 2013b). Based on their phage susceptibility, strains were categorized in to six groups: group 1. Includes the 16 non-plaqueing strains, the other five groups of strains were sensitive to various level of phages (Table 10).

Table 10. Classification of LAB strains based on phage tying results.

Group 1	Group 2	Group 3		Group 4	Group 5		Group 6	
#802	#805	#840	#833	#810	#830	#825	#854	#843
#804	#819	#864	#838	#839	#879	#834	#828	#863
#808	#813	#883	#869	#846	#890	#860	#853	#876
809	#845	#803		#832	#806		#842	#820
#814	#867	#885		#812	#811		#801	#870
#815	#877	#823		#895	#889		#837	
#824	#878	#894			#827		#851	
#836	#886	#861			#868		#849	
#841	#887	#893			#807		#848	
#844	#857	#896			#847		#874	
#850	#821	#816			#888		#818	
#855	#881	#852			#892		#822	
#856	#826	#862			#891		#875	
#865	#829	#858			#873		#817	
#866	#831	#859			#880		#884	
#872		#882			#835		#871	
16	**15**	**19**		**6**	**19**		**21**	

This classification of strains is based on their phage susceptibility, and strains are grouped in order of increasing from the insensitive group 1 to the most sensitive group 6.

A statistical analysis was made to test if phage susceptibility of bacterial strains has any relationship with genes encoding different CWPS type of *L. lactis* strains. Thus Table 8 and 10 are used for this test. And the statistical result is indicated in Table 11.

Table 11. Relationship between phage susceptibility isolates and their CWPS types

CWPS typing of LAB Strains by Multiplex-PCR	Phage susceptibility of LAB strain base on phage typing						Remark
	Group2	Group3	Group4	Group5	Group6	Total	
IL/KF (B)	6	4	4	19	12	45	
MG/SK (C)	6	12	2	0	5	25	$X^2 = 27.0$
IL/KF, MG/SK (B/C)	1	2	0	0	3	6	*P-value* < 0.05
Total	**13**	**18**	**6**	**19**	**20**	**76**	

This table shows the relationship between CWPS encoding genes and phage susceptibility of strains. After the common individual values were plotted into online statistical software mentioned in the material and methods, the test statistic $X^2 = 27.0$ was obtained at degree of freedom (DF) 8 with probability value of 0.001. Moreover, analysis was done including the CWPS negative results, the level of significance was shown to be reduced, but when they are excluded from the analysis, level of significance increased. Moreover, analysis was done by taking only two strains of *L. lactis* CWPS type B and C at a time and their sensitivity to different phage groups was evaluated and statistical results showed that the two CWPS type B and C are significantly associated ($p < 0.000$) with the different phages. However, no correlation was found between host range and CWPS types.

6. DISCUSSION

Phages that attack dairy starter cultures remain the main cause of fermentation failure in the dairy industry and result in significant economic losses. To minimize such economic losses detection of phage in bulk starter and whey is an important factor to prevent infection of LAB during fermentation processes (Kongo, 2007; Garneau & Moineau, 2011; Marco *et al*., 2012). In this study, phages were isolated from bulk starter and whey samples and after propagation of phages on their respective hosts; they were used for phenotypic and genotypic differentiation of strains. The samples tested in this study were collected when fermentation problems was experienced in the cheese making process. We found that high level of phages particularly in the BS samples, however, our phage isolates didn`t cause significant fermentation problem as tested by culture S. This may show that phage detection in mixed starter cultures do not always cause fermentation failure (Kleppen *et al.* 2011), because there may be other strains which substitute the normal fermentative activities.

More than 91 strains isolated from culture S, were sensitive to at least one of the phages isolated in this study. Comparing to previous findinding (Kleppen *et al.*, 2011), who has been isolated low level of infectious phages. In this study high level of phages isolated from samples collected from TINE`s cheese industry in Nærbø. In addition, this was only from the plaque assay methods, even most of the 16 initially non-plaquing strains were attacked by another test using the non-purified phages. Thus, during phage typing of strains, non-plaquing strains were tested many times with both sources of phages; and some strains which were not sensitive in the one test were attacked in another test. Moreover, high number of phage inhibition was shown by growth kinetics study, including strains that were not targeted in the plaque assay.

The susceptibility of bacterial isolates to phage infection showed that there was a huge variation between strains. In some cases phage susceptibility of strains was consistent with results of the molecular methods. For instance, the phage susceptibility of strain #844 and #855 was consistent with their similarity by both PCR methods. These results correlate with these obtained from both molecular methods. Such overlapping results by the three methods lead us to suggest they are the same strains. For determination of host ranges of the phages, forty phages isolated by plaque assay method were used in phage typing of all strains. In this case, most strains showed differences in susceptibility, where only one strain (#870) was

sensitive to all the phages, while about 16 strains were not sensitive to these phages. All phages assessed in this study have a relatively broad host range infecting at least 4 (Φ837) at most 42 (Φ878) different strain hosts. However, this result contradicts with previous report by Rousseau and Moineau (2009), which stated that most LAB phages display a very narrow individual host range. Only few phage isolates obtained from non-purified phages appeared to have a narrow host range, infecting only the bacterial strains from which they were isolated (Fig. 2). This may be explained by the principle of extreme host specificity of phages (Holmfeldt *et al*., 2007). The variation in the phage sensitivity of bacterial strains was different between the two the same methods. Infectivity of LAB strains by the non-purified phages was higher than by the purified phages, this may be regardless of the phage concentration non purified phages may contain more diversity of phage types.

Bacterial strains were further analyzed by $(GTG)_5$-PCR fingerprinting and multiplex PCR. $(GTG)_5$-PCR fingerprinting cluster analysis revealed two major lineages (Fig. 9B). The first lineage (shown as Group 1) contains 5 strains namely, #803, #849, #801, #825 and #826, which have 74% similarity level. While the other second lineage included the rest 91 strains which was differentiated in to 7 groups. However, the resolution by this method was lower than by phage typing. Thus, phage typing method was shown to allow finer discrimination of strains than using the molecular techniques. Our results can be supported by previous research findings (Koehler *et al.,* 1991), which explains for methods that depend on the use of the nucleotide sequences of conserved genes are difficult to design strain-specific probes. Moreover, this may be strengthened by another report (Rademaker *et al*., 2007), which stated that phenotypic differences between lactococcus isolates does not apparently reflected by $(GTG)_5$-PCR. Furthermore, the close relationship between strains of *L. lactis* remained indistinguishable using $(GTG)_5$-PCR genomic fingerprinting analysis (Rademaker *et al*., 2007).

The application of multiplex PCR using three *L. lactis* specific primer pairs targeting specific genes used for diversity analysis and strains were grouped based on their molecular sizes (Table 4). The three primer pairs used in multiplex-PCR were: UC/CV, IL/KF, and MG/SK, they are assumed to detect specific genes encoding CWPS type A, CWPS type B and CWPS type C respectively (Mahony *et al*., 2013b). Thus, the three specific primers indicates that the cell surface of most *L lactis* strains have CWPS, which in turn determines phage sensitivity of those strains (Ainsworth *et al*., 2014). This technique was efficient to detect 95% of the

strains. The ratio of strains with genes encoding the biosynthesis of CWPS group B and C was 2:1. The five strains that were not detected by the CWPS genes typing are supposed to be changed in their structure or mutations in key biosynthetic genes are expected to cause detrimental effects on growth as demonstrated by the formation of abnormally long-chained lactococci carrying mutations in CWPS synthesis genes (Dupont *et al*., 2004). Although, multiplex PCR separated majority of the bacterial isolates as strains of *L. lactis,* most of them showed a unique pattern of susceptibility to the tested phages (Fig. 4). The CWPS negative results were small in number, but their presence was shown to affect the association between phage susceptibility and CWPS types, thus strong association was found between phage susceptibility and CWPS types. Our results are also consistent with previous finding by Shibata *et al*. (2009), which stated that most bacteriophages infecting LAB recognize cell wall polysaccharides.

The primary source of phages which always appear in dairy plants is not clearly known. However, various sources of phages in cheese production have been indicated (Garneau and Moineau, 2011). Phages may persist in raw milk (Marco *et al*., 2012), and phage in remained liquids such as whey and other in-house sources associated with inadequate sanitation (Madera *et al*., 2004; Kleppen *et al*., 2011; Marco *et al*., 2012). In this study, we used two methods of phage typing using 40 purified phages obtained from 40 plaques, and another 40 non-purified phages from the spots of the non-plaquing strains previously stamped with the BS samples. After typing of bacterial strains with both types methods, it was found that the two preparations have different spectrum of activity (Table 2 and 4). It may show that a BS sample contains a large number of phage variants and more than 96 bacteria strains that may be important for cheese making.

Since it is impossible to avoid phages from dairy environments, phage control strategies are designed to reduce their impacts rather than eradicate them. Culture rotation, direct vat inoculation of starters, avoiding production of bulk starter cultures, careful handling and disposal of whey, use of phage-inhibitory media, and maintaining of optimized sanitation are some of the recommended practices to minimize phage spreading in dairy plants (Coffey and Ross, 2002; Moineau and Levesque, 2005; Kleppen *et al*., 2011).

Finally using a mixture of phages fermentation inhibition test was performed. In this experiment, the effect of phages on fermentation activity of mixed starter cultures was studied using a pool of phages prepared from the 40 phages isolated from this study and 39 phages

(from Cyril Frantez) inoculated in to culture S. The first two treatments groups (Mix 1 and Mix 2), starter cultures showed a slight reduction in acid production lower than 10%, which we suggested that this may not good indication for the effect of virulent phages in these samples (Harrigan,1998; Oriani and Yokoya, 2004). On the other hand, Mix 3 and Mix 4 treated cultures showed 0.22 and 0.21 of loss in fermentative activity. Though, several factors can affect starter cultures` ability to acid production (Oriani and Yokoya, 2004), except in one treatment group, lytic activities of phages were not shown to cause significant reduction in pH than the controls. The maximum reduction in fermentation activities of starter cultures was shown by the highest number of phages. However; the virulence nature of phages isolated in this study, which was shown on plaque assay, did not cause major fermentation failures. It was suggested that, the effect of phages on mixed starter cultures may not be informative for the virulence nature of the phages; this result may indicate the presence of phage-insensitive strains continued to grow and acidify (Kleppen *et al*., 2011; Marco *et al.*, 2012). Thus, at this level we have not sufficient evidence to generalize about the real effect of phages on carbohydrate fermentation. Therefore, further studies using single starter cultures are required to reveal the actual impact of the isolated phages on dairy fermentation processes.

7. CONCLUSIONS

This study shows that there is a huge variation in phage susceptibility even within closely related strains; similarly isolated phages have broad host range diversity. This study shows also large number of phage variants in a bulk starter, more than the 96 bacterial strains. The phage susceptibility of strains was also highly variable. Differences among bacterial isolates shown by plaque assay were not reflected by genotypic methods, showing that phage typing have high resolution than the used genotypic methods. $(GTG)_5$ primer is fast to detect bacterial strains but lacks to discriminate between closely related subspecies and strains. Furthermore, CWPS typing of bacterial isolates was crucial in differentiation of strains. Such grouping could be used as a fast approach to differentiate collection of bacterial strains. Our results showed that majority of the isolated strains were found to be member of *Lactococcus lactis* with CWPS type B and type C. Furthermore, this study also identified there is strong relationship between phage susceptibility of the strains and their CWPS genes. However, all the strains, phage type and molecular nature of their receptor binding proteins need to be characterized in detail.

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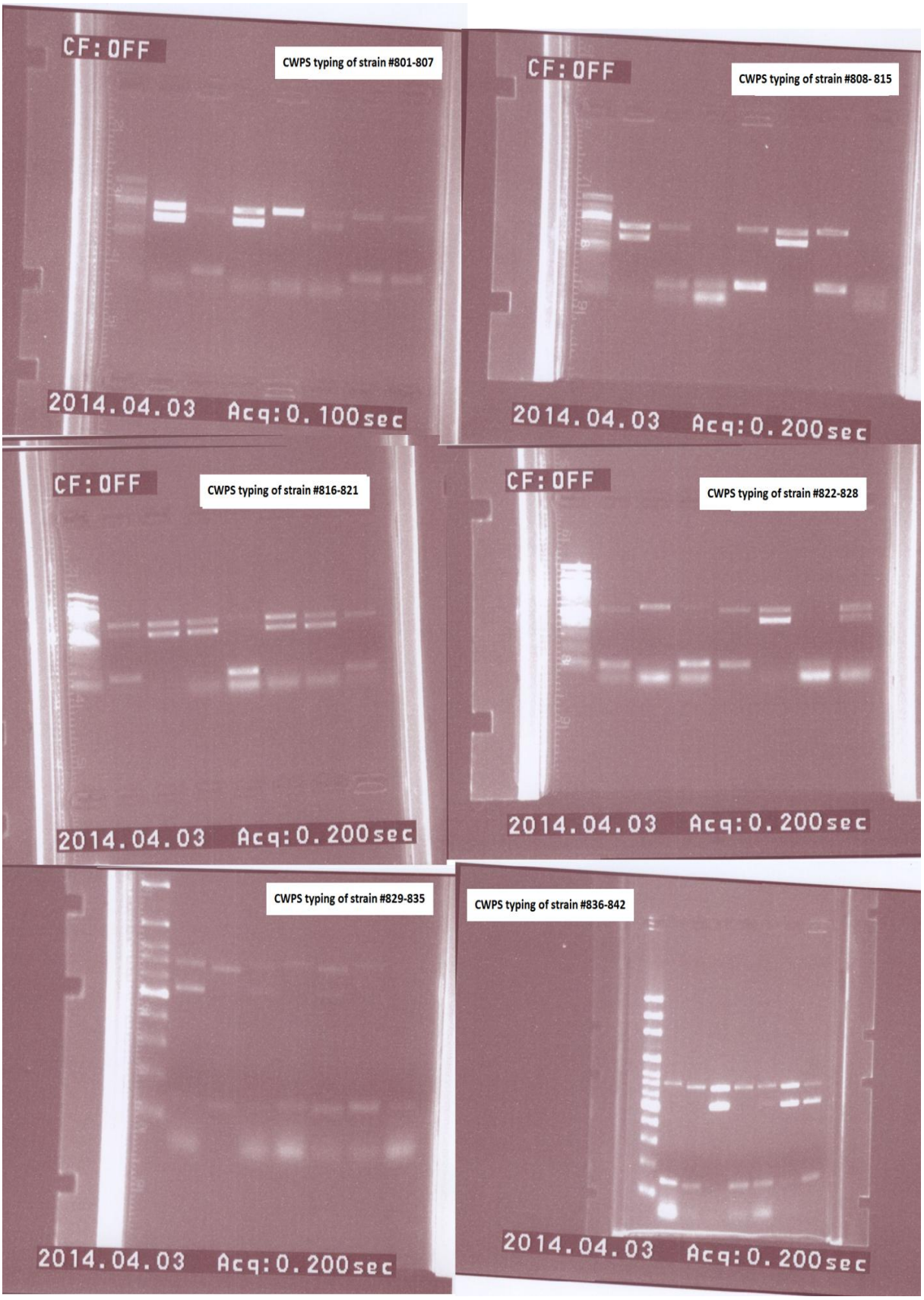
APPENDIXES

Appendix 1. Phages used in acidification test

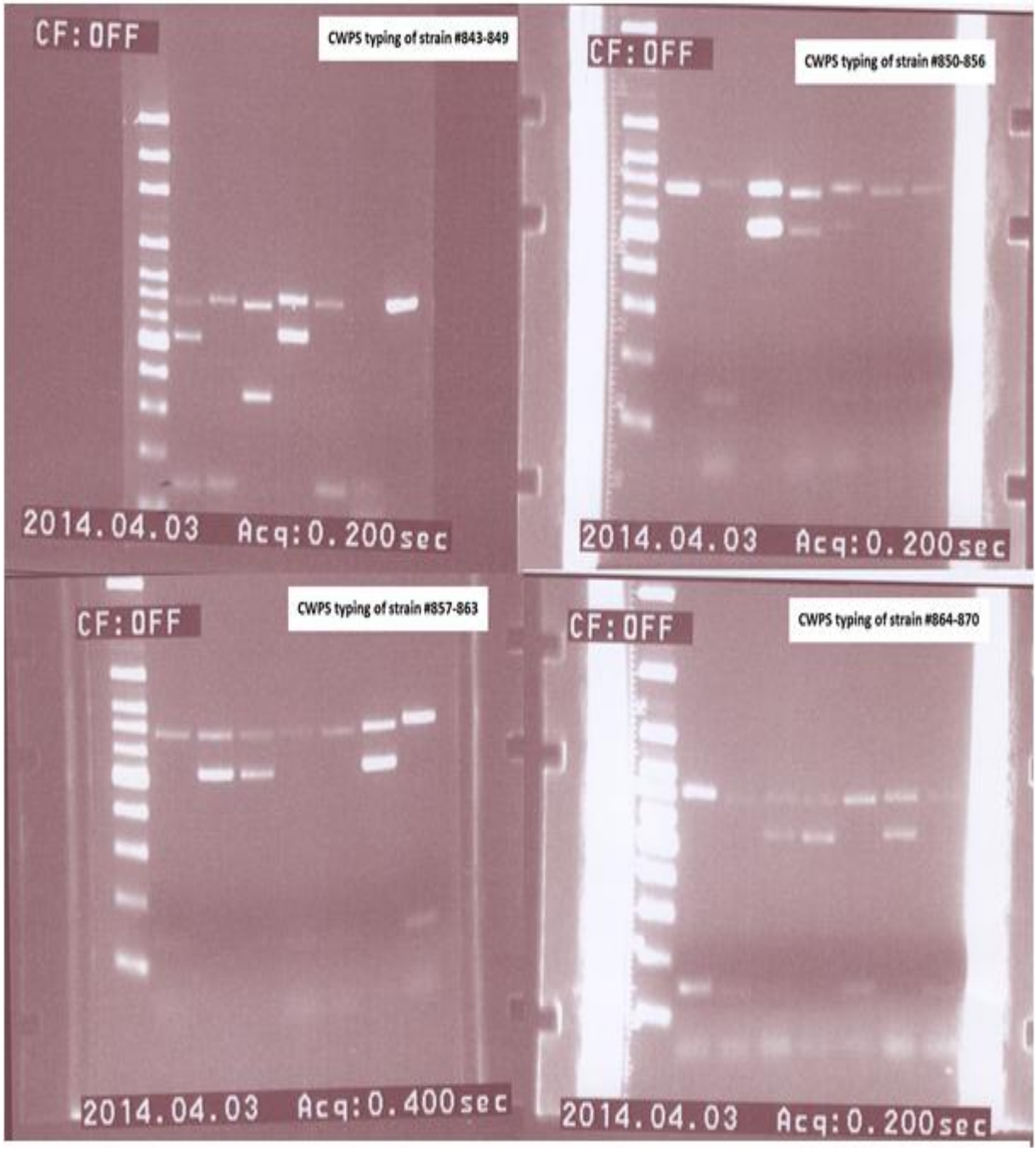
Mix 1	Mix 2	Mix 3	
Φ8786 (42)	Φ837	811	ΦD3
Φ848 (38)	Φ838	Φ813	ΦD4
Φ831(37)	Φ827	ΦB10	ΦF1
Φ815(15)	Φ811	ΦB3	ΦF10
Φ862(18)	Φ817	ΦB4	ΦF2
Φ864(38)	Φ888	ΦB5	ΦF3
Φ847(38)	Φ813	ΦB6	ΦF4
	Φ815	ΦB7	ΦF7
	Φ818	ΦB8	ΦF8
	Φ861	ΦC10	ΦG1
	Φ867	ΦC11	ΦG12
	Φ889	ΦC3	ΦG2
	Φ819	ΦC4	ΦG3
	Φ820	ΦC6	ΦG4
	Φ822	ΦC7	ΦG5
	Φ886	ΦC8	ΦG6
	Φ887	ΦD1	ΦG7
	Φ864	ΦD11	ΦG8
	Φ890	ΦD12	ΦH4
	Φ868	ΦD2	ΦH5
	Φ862		
6	21	40	

Appendix 2A. Shows band patterns of CWPS typing of strains by Multiplex PCR

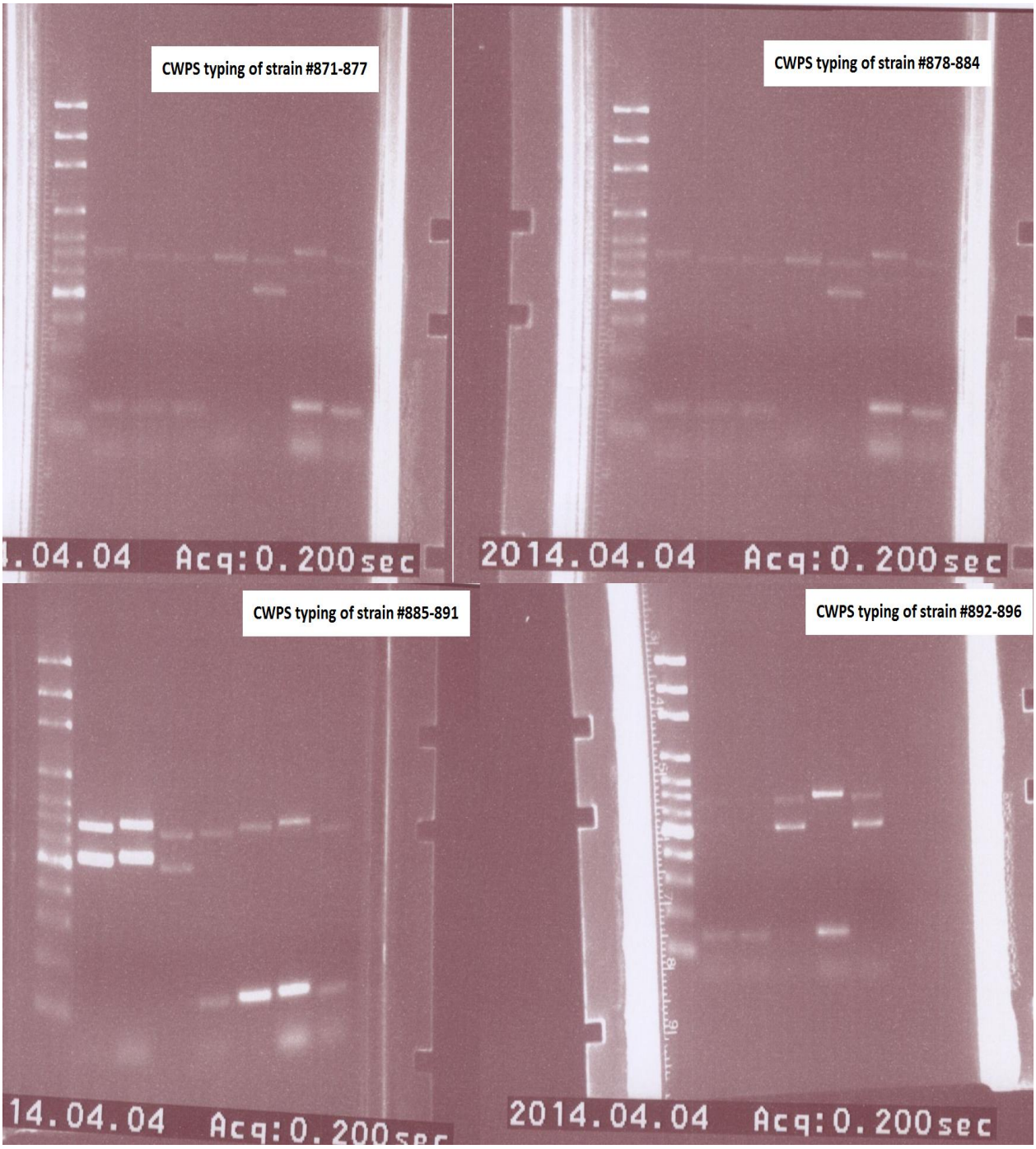


Fig· Shows CWPS typing of LAB strains #801 to #842

Appendix 2B. Shows band patterns of CWPS typing of LAB strains by Multiplex PCR



Appendix 2C. Shows band patterns of CWPS typing of strains by Multiplex PCR



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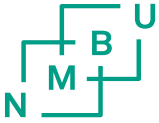
Appendix 3. A scheme used during growth kinetics.

Plate 1	Each row contains 6 control samples	List of 6 phage treated samples in each row
A	A1-A6 (#801-806)	A1-A6 (#801-806)
B	B1-B6 (#813-818)	B1-B6 (#813-818)
C	C1-C6 (#825-830)	C1-C6 (#825-830)
D	D1-D6 (#837-842)	D1-D6 (#837-842)
E	E1-E6 (#849-854)	E1-E6 (#849-854)
F	F1-F6 (#861-866)	F1-F6 (#861-866)
G	G1-G6 (#873-878)	G1-G6 (#873-878)
H	H1-H6 (#885-890)	H1-H6 (#885-890)
Plate 2		
A	A7-A12 (#807-812)	A7-A12 (#807-812)
B	B7-B12 (#819-824)	B7-B12 (#819-824)
C	C7-C12 (#825-836)	C7-C12 (#825-836)
D	D7-D12 (#843-848)	D7-D12 (#843-848)
E	E7-E12 (#855-860)	E7-E12 (#855-860)
F	F7-F12 (#867-872)	F7-F12 (#867-872)
G	G7-G12 (#879-884)	G7-G12 (#879-884)
H	H7-H12 (#891-896)	H7-H12 (#891-896)

Initially each LAB starter strain was labelled with letter, thus some of the results of plaque assay are indicated with letter.



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