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Original article

INHIBITORY EFFECT OF CASEIN AND ALPHA-LACTALBUMIN ON *CHRYSEOBACTERIA SPP.* ISOLATED FROM MILK AND DAIRY PRODUCTS

N. A. DINA & W. M. ELSHERIF

Certified Food Laboratory, Animal Health Research Institute,
Agriculture Research Center, Egypt

Summary

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In this study, pathogenic and food spoilage bacteria *Chryseobacteria* spp. were isolated by a new method from 150 samples of raw milk (26/50), heat-treated milk (HTM) (5/50) and butter (2/50). The species with the highest prevalence was *C. indologenes* (10.67%) followed by *C. bovis* and *C. gleum* (4.67% each), and *C. bernardetii* (2%). The three new strains *C. bernardetii, C. gleum*, and *C. indologenes* were named MW703610, MW703611, and MW703612, respectively, with identities ranging from 97.8% to 98.9%. Gene sequencing was performed on some isolates and proved the highest sequence similarity for *C. bernardetii* WD1 (97.8%)*, C. gleum* WD2 (98.9%) and *C. indologenes* WD3 (98.9%). Most of the isolated *Chryseobacteria spp.* showed proteolytic and lipolytic activity. Therefore, casein and alpha-lactalbumin were used as natural antibacterial substances against all isolates. Higher bioactivity against the isolates was obtained using casein rather than the alpha-lactalbumin. Based on these results, casein and alpha-lactalbumin may be promising natural bioactive agents against pathogenic *Chryseobacteria* spp. Further studies should be done to establish the natural peptide fractions associated with the observed activity.

Key words: alpha lactalbumin, casein, *Chryseobacteria* spp., proteolytic and lipolytic activity

INTRODUCTION

The genus *Chryseobacterium* is a member of the family *Flavobacteriaceae* (phylum Bacteroidetes) and currently consists of 21 species. Members of the genus are Gram-negative, aerobic, non-fermentative, oxidase-positive, catalase-positive, non-motile, yellow-coloured, rod-shaped, non-spore-forming bacteria (Izaguirre-Anariba & Sivapalan, 2020; Zhang *et al.*, 2020). They are ubiquitous and have been isolated from a wide variety of habitats including plants, soil, roots of trees, clinical specimens, animal bodies, freshwater, wastewater, compost, sludge, sediments, and dairy products (Nishioka *et al*., 2016; Pal *et al*., 2018; Lee *et al*., 2019). Al-

though they are considered innocuous environmental contaminants colonising washbasins and faucets in the clinical environment, they may serve as a potential source of infection for neonates and immune-compromised individuals (Izaguirre-Anariba & Sivapalan, 2020). They have been described as etiological agents of meningitis, bacteraemia, pneumonia, endocarditis, infections of the skin and soft tissues, ocular infections, etc. (Kirby *et al*., 2004). *Chryseobacterium* spp. members have also been reported to be a part of psychrotolerant and proteolytic bacterial population that causes a variety of problems in food products such as milk, milk products, meat, poultry and fish (Hantsis-Zacharov *et al*., 2008). However, the most important spoilage of ultra-heat treated (UHT) milk, sterilised milk and related UHT dairy products e.g. cream, custard, evaporated condensed milk, chocolate milk, flavoured milk, infant formula and drinks based on milk is caused mainly by enzymes of bacterial origin that resist the UHT treatment (Machado *et al*., 2017).

Milk contains many antimicrobials including immunoglobulins from the mother's blood to protect neonates from infection. Lactoferrin and lysozymes form complexes and seclude iron while the lactoperoxidase interferes with microbial cell walls enhancing their porosity (Omara, 2019). Cow casein and alpha-lactalbumin are the most widely used sources for antimicrobial and antioxidant molecules in the agricultural and food industries. They are isolated isoelectrically and by filtrate thermo-precipitation at 75 °C (Lajnaf *et al*., 2021).

The calcium-casein phosphoprotein assembly that clumps in clusters as micelles gives milk its opaque white colour. Heat denaturation does not affect the structurally disordered casein fraction. Casein micelles include hydrophobic and hydrophilic areas in their amino acid sequences, and the assemblage is a multi-dispersed surfactant system comprising spheroidal aggregates with quadratic subunits with molecular weight, isoelectric point (pI) and phosphate groups that differ greatly. The quadrature is strictly only electrophoretically differentiable as alpha s1 (αs1), alpha s2 (αs2), beta (β) and kappa (κ) caseins in order of their decreasing degree of motion at pH 7.1 (Horne, 2009). The addition of acid to casein causes colloidal calcium hydroxyl phosphate in the casein micelle to be dissolved and solubilised as in natural milk souring. Whey proteins, the filtrate remaining after casein isolation from skimmed milk, are a heat-labile family of globular milk proteins composed predominantly of lactoferrin (LF), beta-lactoglobulin (β-lg), alpha-lactalbumin (α-la), serum albumin (SA), immunoglobulins (IGs), folate-binding protein, lactoperoxidase, transferrin, ferritin, protease peptone, calmodulin (calcium-binding protein) and prolactin. Alpha-lactalbumin is the predominant whey protein in raw cow milk (Inglingstad *et al.,* 2010).

The present study was designed primarily to determine the incidence of lipolytic and proteolytic activities of *Chryseobacteria spp.* isolated from raw milk and some dairy products. A second goal was to register the new sequence genes in GenBank and finally, it aimed to investigatethe *in vitro* activity of cow casein and alpha-lactalbumin against the isolated *Chryseobacteria* spp.

MATERIALS AND METHODS

Collection of samples

A total of 150 samples of raw milk, heat-treated milk (HTM) and butter samples

(50 samples each) were collected from dairy shops in different locations in Assiut city, Egypt. Samples were brought to the laboratory using refrigerated transport, within a maximum time frame of 2 h, and prepared according to APHA (2004). Briefly, each sample of raw and heat-treated milk was thoroughly mixed, while each of butter samples was left to melt in a thermostatically controlled water bath at 40 °C for no more than 15 min. Then, the sample was thoroughly mixed and homogenised by a sterile stirrer before being examined.

Isolation and identification of Chryseobacteria spp.

It was performed as per Nishioka *et al.* (2016). Five mL or grams of prepared samples were added to 10 mL nutrient broth, which was supplemented with tobramycin (1 mg/L) (as an enrichment step) and then incubated at 25±2 ^{o}C for 48 h. After that, broth-loaded samples were inoculated onto R2A agar media supplemented with cycloheximide (50 mg/L) and tobramycin (1 mg/L) (R2A-C/T) and incubated at 25±2 ^{o}C for 7–10 days. At the end of the incubation period, bacteria with a yellow-orange colour were isolated and confirmed biochemically using the method described by Hantsis-Zacharov *et al.* (2008).

PCR for confirmation for isolates

This part of the work was performed at the Reference Laboratory for Veterinary Quality Control, Biotechnology Unit in Animal Health Research Institute, Dokki, Giza, Egypt. Preparation of bacterial DNA, PCR amplification and sequencing of the 16S rRNA gene were carried out as described by Lagacé *et al.* (2004). PCR products were purified using QIA quick PCR product extraction kit (Qiagen, Hilden, Germany). Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) was used for the sequence reaction and then purification was done using the Centrisep spin column. DNA sequences were obtained by Applied Biosystems 3130 genetic analyzer (HITACHI, Japan), a BLAST® analysis (Basic Local Alignment Search Tool) (Altschul *et al.*, 1990) was initially performed to establish sequence identity to the GenBank entries. The sequences of strains MW703610, MW703611 and MW703612 were manually aligned against sequences obtained from the GenBank database. The phylogenetic tree was created by the MegAlign module of Lasergene DNAStar version 12.1 (Thompson *et al*., 1994) and phylogenetic analyses were performed using maximum likelihood, neighbour-joining and maximum parsimony in MEGA6 (Tamura *et al*., 2013).

Lipolysis and proteolytic activity of isolated strains

Lipase activity was evaluated as followed. One hundred milliliters of Trypticase Soy Agar (BBL 211043) was supplemented with 1 mL of Tween 80 (Fluka 93781) to serve as a substrate in this test. After inoculation, samples (bacteria) were incubated at 25 ^{o}C for 72 h. The appearance of a turbid halo around a colony was taken to indicate positive result (Janda & Bottone, 1981; Edberg *et al*., 1996; Pavlov *et al*., 2004).

For proteinase activity detection, skim milk powder (L31, Oxoid, UK) was incorporated into dialysed Brain Heart Infusion broth (CM0375, Oxoid) with the addition of 1.5% agar. After inoculation, the plates were incubated for 72 h at 25 ^{o}C. The formation of a clear zone around the colonies indicated positive result (Edberg *et al*., 1996).

Preparation of casein and alpha-lactalbumin

Casein and alpha-lactalbumin were prepared according to the methods reported by Omara (2019) and Richter *et al*. (1973) with minor modification. In brief, skimmed cow milk (the plasma phase of milk) was prepared by centrifugation of pooled whole milk samples at 5000 rpm for 15 min. Samples of 250 mL skimmed milk were placed in 500 mL beakers and warmed in a water bath to bring the temperature to 40 °C. To the warmed skimmed milk, 1 M acetic acid solution was added drop by drop with constant stirring until a pH of 4.6 (the isoelectric point) was attained. The beakers were kept in a water bath at 40 °C until no observable precipitation occurred and the milk was subsequently filtered twice through a cheesecloth. The residues were labelled as casein. The obtained filtrates were heated to 50 °C for 5 min to produce precipitates that were filtered from the hot solutions through Whatman filter paper No.1 (Sigma-Aldrich, St. Louis, MO, USA). Polyethylene glycol (PEG) was ground to a fine powder in a mortar and added slowly with mixing to whey (at 4 °C) to a final concentration of 30% (w/v). The resulting solution was held at 4 °C for 24 h after which the precipitated whey proteins were removed by centrifugation at 20,000 rpm for 20 min. The resulting PEG supernatant was adjusted to pH 7.8 and re-centrifuged to remove mainly inorganic salt precipitate. Twenty-five milliliters of this latter supernatant were diluted with an equal volume of Tris-HCl buffer (0.075 M, pH 7.8) to precipitate all proteins except the alpha-lactalbumin and this process was repeated until clear whey was obtained. The Tris-HCl buffer was eluted with a linear 0.15 M NaCl gradient in the same buffer to produce cow milk alpha-lactalbumin.

Antibacterial activity of casein and alpha-lactalbumin

It was assessed using the agar well diffusion method. *Chryseobacteria* spp. suspensions were prepared and compared to a concentration of 0.5 McFarland standard. A swab was used to spread 0.1 mL of suspension on solidified Mueller Hinton agar plates with 4 mm diameter wells in the agar. Each well was filled with 80 µL of previously prepared casein and alpha-lactalbumin. All plates were incubated at 25 ± 2 ^{o}C for 24 h.

Statistical analysis

Statistical analysis of the antibacterial activity of casein and alpha-lactalbumin was carried out by using the SPSS program (SPSS Inc., Chicago, IL, USA).

RESULTS

Chryseobacteria spp. were isolated from 52% of local raw milk samples, 10% of HTM and 4% of butter samples. The most frequent species in all samples were *C. indologenes* (10.67%) followed by *C. bovis, C. gleum* (4.67% each), and *C. bernardetii* (2%) (Table 1). In raw milk, 24% of samples carried *C. indologenes*, 10% had *C. bovis,* 12% had *C. gleum* and 4% had *C. bernardetii.* However, only *C. indologenes* (4%), *C. bovis* (4%) and *C. gleum* (2%) could be detected in HTM. Butter samples had only *C. indologenes* in 4% of samples (Table 1, Fig. 1).

Confirmed colonies were selected for further characterisation by 16S rRNA gene sequence analysis at 1485 bp as shown on Fig. 2.

Preliminary sequence comparisons with 16S rRNA gene sequences held in

Table 1. Incidence of *Chryseobacteria* spp. isolated from the raw milk and some dairy products

Chryseobacteria spp.	Raw milk (n=50)		HTM (n=50)		Butter (n=50)		Total (n=150)	
	number	%	number	%	number	%	number	%
C. bovis	5	10	2	4	0	0	7	4.67
C. indologenes	12	24	2	4	2	4	16	10.67
C. gleum	6	12	1	2	0	0	7	4.67
C. bernardetii	3	6	0	0	0	0	3	2.00
Total	26	52	5	10	2	4	33	22.00

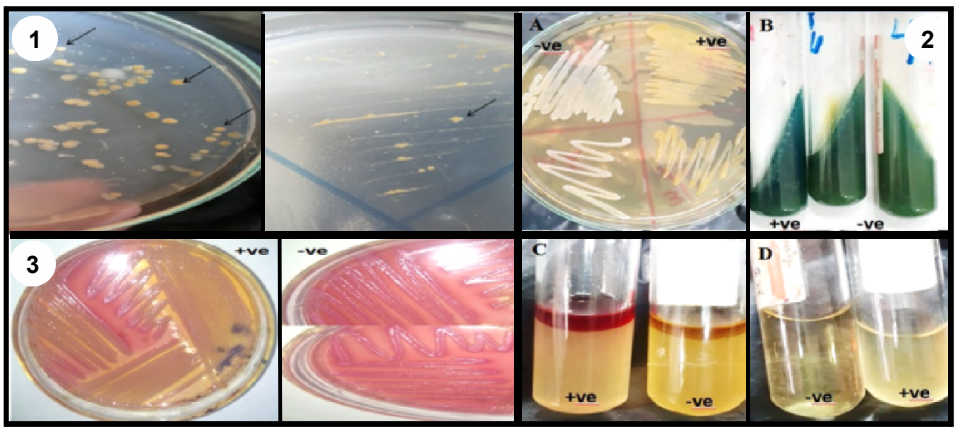

Fig. 1. **1**). Yellow pigmented colonies of *Chryseobacteria* spp. on R2A agar; **2**). Some of biochemical tests of isolated *Chryseobacteria* spp.: growth on Mueller Hinton agar (A); Simon's citrate test (B); indole test (C); growth at 3% NaCl (D); **3**). MacConkey agar test.

Gene Bank indicated that strains were closely related to the genus *Chryseobacterium*. The newly determined sequences were then aligned with phylogenetic tree constructed using Gene Bank data and the highest 16S rRNA gene sequence similarity for MW703610 was *C. bernardetii* WD1 (97.8%), for MW703611 was *C. gleum* WD2 (98.9 %) and for MW703612 *C. indologenes* WD3 (98.9%). The sequence similarities with respect to all other species of the genus *Chryseobacterium* were below 96.0%.

The Neighbour-joining analysis and maximum parsimony in MEGA6 confirmed that MW703610, MW703611 and MW703612 were phylogenetically most closely related to members of the genus *Chryseobacterium*. The tree based on the MegAlign method had essentially the same topology as shown in Fig. 3 and 4. The complete genome sequences were deposited in GenBank under the accession numbers SUB9206722 Seq1 MW703610, SUB9206722 Seq2 MW703611 and SUB9206722 Seq3 MW703612.

Table 2 shows that three of *C. indologenes* from raw milk and one from butter and one isolate of *C. bernardetii* from raw milk produced lipase. Proteolytic activity was demonstrated by 4 *C. bovis* isolates from raw milk and 2 from HTM,

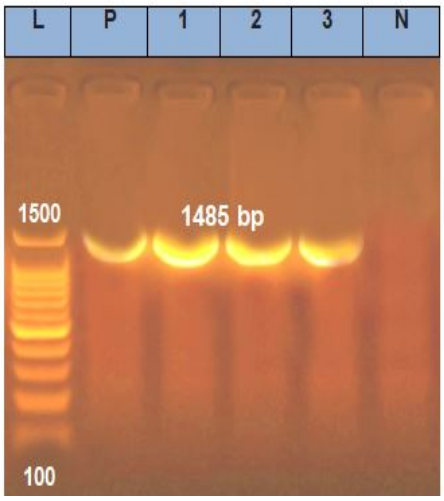

Fig. 2. Amplified *Chryseobacteria* spp. 16S rRNA gene recovered from dairy products. L: molecular marker; lane P: positive control; lane N: negative control; lanes 1–3: positive isolates.

9 isolates of *C. indologenes* from raw milk, one from HTM and one from butter, 5 *C. gleum* isolates from raw milk and HTM and one *C. bernardetii* isolate from raw milk (Fig. 5).

Table 3 showed significant differences in antibacterial activity of the prepared casein and alpha-lactalbumin against different *Chryseobacteria* spp. The mean inhibition zone of casein was the highest in *C. gleum* (4.17 cm) while in *C. bovis*, *C. indologenes* and *C. bernardetii* the respective zones were 3.53, 2.05 and 3 cm, respectively. The mean inhibition zone of alpha-lactalbumin was the highest in *C. bovis* (3.57 cm) while in *C. gleum*, *C. indologenes* and *C. bernardetii* zones were 3.12, 1.92 and 2.15 cm, respectively (Fig. 6).

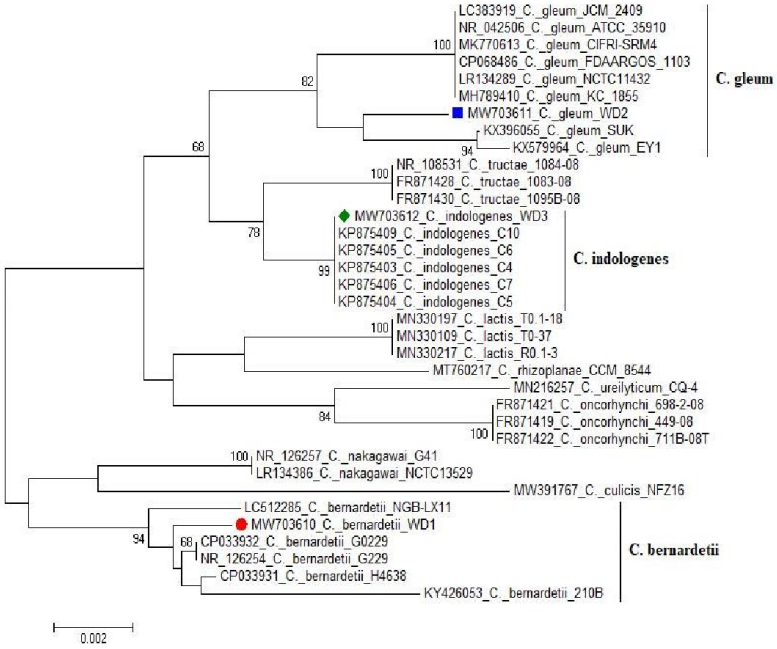

Fig. 3. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the position of strains members of the genus *Chryseobacterium* and representatives of related taxa. Maximum-likelihood unrooted tree generated after 500 bootstraps indicated clustering of the tested strain with strains apart of *Chryseobacteria* spp.

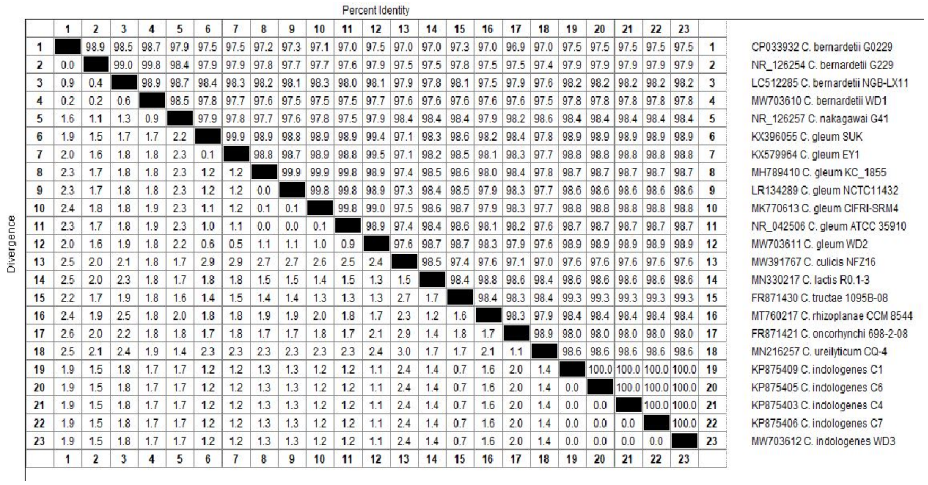

Fig. 4. Sequence distance of the *Chryseobacterium* strains (generated by laser gene software) showing identity range of 97.8-98.9%.

Table 2. Lipolysis and proteolysis activities of the isolated *Chryseobacteria spp.*

Types of samples	Positive lipolysis activity			
	C. bovis	*C. indologenes*	*C. gleum*	*C. bernardetii*
Raw milk	0	3	0	1
Heat-treated milk	0	0	0	0
Butter	0	1	0	0
	Positive proteolysis activity			
	C. bovis	*C. indologenes*	*C. gleum*	*C. bernardetii*
Raw milk	4	9	5	2
Heat-treated milk	2	1	1	0
Butter	0	1	0	0

DISCUSSION

Gram-negative psychrotrophic pathogenic bacteria like *Chryseobacterium* spp. thrive in milk and milk products. Human diseases, food illness, and food deterioration have all been linked to milk and milk products (Mariam, 2021). *Chryseobacteria* spp. have been isolated from a variety of foods and environments and are recognised to be human pathogens (Hantsis-Zacharov & Halpern, 2007; Hantsis-Zacharov *et al.,* 2008; Tsôeu *et al*., 2016; Yoon *et al*., 2019; & Fusco *et al*., 2020). *Chryseobacteria* spp. can be found in milk and dairy products from a variety of sources, including animal feed, the environment (air, water, barn, and pasture), the udder and milking equipment of cows, and during storage and transportation.

In this study, the identification of *Chryseobacteria* spp. was based on phenotypic characterisation using a new technique to improve the recovery rate of target microorganisms on selective medium (R2A/TC) that was reported as a specific medium for the isolation of *Chryseobacteria* spp. (Nishioka *et al*., 2016). The nutri-

ent broth is a basic medium for isolation of bacteria in general microbiology. It used as an enrichment broth for the isolation of *Chryseobacteria* spp. by adding tobramycin, which acts as a strong inhibitor for most Gram-negative bacteria, with no effect on the *Chryseobacterium* spp. (Christakis *et al*., 2005; Maravić *et al*., 2013; Khianngam *et al*., 2014). An antifungal agent such as cycloheximide was

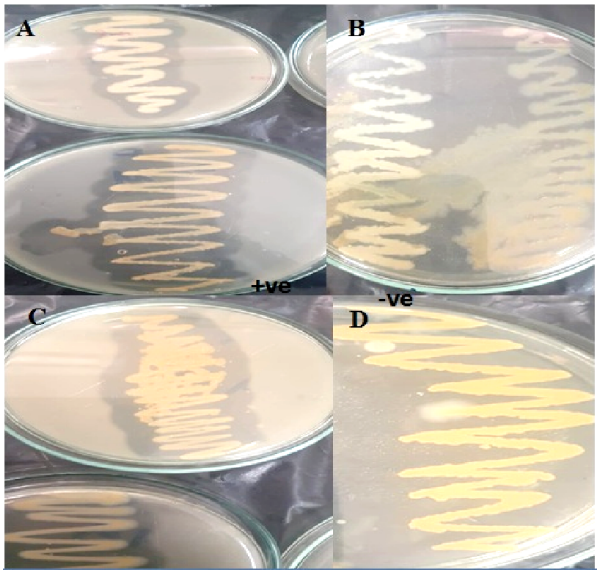

Fig. 5. Proteolytic and lipolytic ability of isolated *Chryseobacteria* spp. **A**: Positive zone for proteolytic activity; **B**: No zone (negative) for proteolytic activity; **C**: Positive zone for lipolytic activity; **D**: No zone (negative) for lipolytic activity.

Table 3. Antibacterial activity (zone of inhibition, cm) of prepared casein and alpha-lactalbumin against different *Chryseobacteria* spp. isolates

Chryseobacteria spp.	Casein	
	Number (% positive)	Mean ±SEM (min–max)
C. bovis (n=7)	7/100	3.53 ± 0.03 (2.5–5)
C. indologenes (n=16)	11/68.75	2.05 ± 0.04 (0–5)
C. gleum (n=7)	7/100	4.17 ± 0.06 (2–5.8)
C. bernardetii (n=3)	3/100	3 ± 0 (3–3)
Chryseobacteria spp.	Alpha-lactalbumin	
	Number (% positive)	Mean ±SEM (min–max)
C. bovis (n=7)	7/100	3.57 ± 0.03 (2.5–4.9)
C. indologenes (n=16)	11/68.75	1.92 ± 0.03 (0–5)
C. gleum (n=7)	5/83.3	3.12 ± 0.08 (0–5.8)
C. bernardetii (n=3)	3/100	2.15 ± 0.02 (2–2.3)

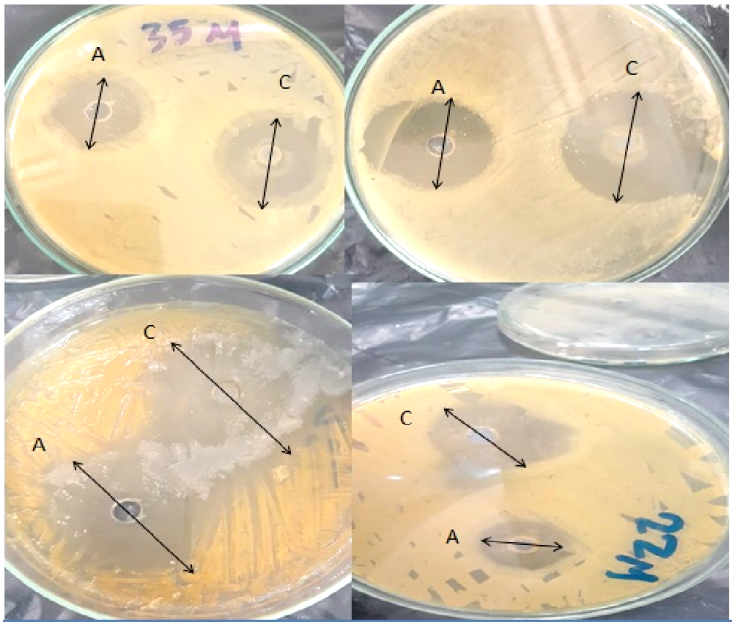

Fig. 6. Antibacterial efficacy of casein (C) and alpha-lactalbumin (A) against isolated *Chryseobacteria* spp.

not required because of the short incubation period of the enrichment step (two days),. However, by using R2A agar and due to long period of incubation (7−10 days), both tobramycin and cycloheximide were added for their strong inhibition of bacteria and a wide range of fungi and yeasts (Nishioka *et al*., 2016; Zhang *et al.*, 2020).

Only yellow or orange-pigmented colonies which changes in colour to flexirubin-type pigments when subjected to KOH, were confirmed biochemically according to Bernardet *et al*. (2006), Hantsis-Zacharov *et al*. (2008) and Cho *et al*. (2010). In this study, based on the polyphasic approaches including phenotypic characterisation, genomic comparison, sequence analysis of 16S rRNA and three housekeeping genes *Chryseobacterium* species (MW703610, MW703611 and MW703612) were phylogenetically most closely related to members of the genus *Chryseobacterium*, isolated from dairy products.

Cold-tolerant bacteria are notorious contaminants of milk and dairy products in the refrigerated dairy food chain (Elsherif *et al.,* 2021). These organisms, especially *Chryseobacteria* spp., may produce heat-resistant enzymes that are responsible for the destruction and decomposition of proteins and lipids in milk and dairy products (Samaržija *et al*., 2012). Such biochemical reactions result in a variety of defects in raw or unprocessed milk, which may affect the suitability of such milk for further processing and consumption. The enzymes produced may cause defects in long-life dairy products such as cheese, butter and long-life milk. In the present study, all strains of yellow-pigmented psychrotrophic bacteria, *Chryseobacteria* spp., isolated from local dairy products and tested for their ability to produce lipase and protease en-

zymes which in turn clarified the economic importance of isolated strains in locally available dairy products (Chen *et al*., 2003). The proteolytic enzymes of *Chryseobacteria* spp. are resistant to pasteurisation and cause bitter flavours, gelation of long-life HTM and responsible for the production of off-odours (Venter *et al.,* 1999). Esias (2008) reported that 57.1% of *Chryseobacteria* spp. had the ability to produce lipase, while all species produced proteinase. Tsôeu *et al*. (2016) found that the practical importance of dairy flavobacteria lies in their psychrotrophic growth and consequent proteinase production in refrigerated milk via poorly sanitised pipelines and equipment.

Casein and alpha-lactalbumin are natural antibacterial biopeptides generated from bovine milk by isoelectric and filtrate thermo-precipitation at 75 °C (Hayes *et al.*, 2006; Omara, 2019). They are broad-spectrum antibacterial compounds especially active against Gram-negative bacteria and are a promising natural treatment for many diseases. In this study the effect of casein and alpha-lactalbumin on different isolated species of *Chryseobacteria* spp. were studied. Statistical analysis indicated that there was no significant difference between the antimicrobial activities of casein and alpha-lactalbumin on the isolated species. However, Omara (2019) reported that alpha-lactalbumin hydrolysates have a higher antibacterial activity than the corresponding casein hydrolysates. Most antibacterial peptides are positively charged and thus electrostatically bound to the negatively charged components of bacterial cell walls, potentially leading to cell destruction (Zhao *et al.*, 2010).

Caseins have been used directly in the food industry not as a by-product, but as an essential ingredient. However, in some dairy processes a surplus can exist and is recovered as a potential co-product. These proteins are usually used as ingredients in various food products to enhance their physical, functional and nutritional properties. They interact well with other molecules having stabilising and emulsifying properties and water-binding capacity. Caseins are inexpensive, non-toxic, biodegradable and highly available (Horne, 2009; Rezaei *et al.*, 2019).

Alpha-lactalbumin is a milk protein that constitutes over one-third of the total protein in human milk. Through recent advances in food processing, pure alpha-lactalbumin has become commercially available, generating new opportunities to research the use of alpha-lactalbumin in nutritional and medical applications and in creating functional food products. Alpha-lactalbumin is an attractive protein because of its physical characteristics, which include a clean flavour profile, high water solubility, and heat stability. These combined properties allow for diverse food applications (Donald *et al.*, 2018).

CONCLUSIONS

Chryseobacteria spp. were isolated in different percentages from milk, HTM and butter samples using R2A agar media supplemented with cycloheximide and tobramycin after enrichment. The most prevalent species was *C. indologenes* followed by *C. bovis, C. gleum*, and *C. bernardetii*. The isolates were subjected to 16S rRNA gene sequence analysis and sequence identity to GenBank accessions. The sequences of strains C. *bernardetii* WD1, C. *gleum* WD2 and *C. indologenes* WD3, were deposited in GeneBank. Most of the isolated strains produced lipase and protease enzymes. All isolates had a significant anti-*Chryseobacteria* activity with

clear, wide zones apparent in the assay. A safe, abundant, high-quality milk supply should be the goal of every dairy producer in the world. To achieve this goal, control strategies start at the farm and continue throughout processing. To meet increased standards for raw milk quality, producers must adapt and apply practices that reduce bacterial contamination and the spoilage of raw milk and dairy products.

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Correspondence:

Walaa M. Elsherif
Certified Food Laboratory,
Animal Health Research Institute,
Agriculture Research Center, Egypt
e-mail: me.elsherif@yahoo.com