Significant Industrial Properties of *Enterococcus faecium* SFD as a Probiotic and Bacteriocin-Producing Strain

Sahar F. Deraz*, Mohammed G. Shehata, Ashraf A. Khalil

Department of Protein Research, Genetic Engineering and Biotechnology Research Institute, City of Scientific Research & Technological Applications, New Borg El-Arab City, P.O. Box: 21934 Alexandria, Egypt
Email: sahar_deraz@hotmail.com

Abstract: Lactic acid bacteria (LAB) have long been used in the production of fermented sausages and other meat-derived commodities. The purpose of the present study was to determine the technological properties of the newly isolated *E. faecium* SFD from homemade Egyptian kareish chesse with potential application biopreservatives and probiotic. The strains were studied to evaluate the effects of different ingredients e.g. salt, nitrite, water activity and fat, on the functionality of the strain as well as nitrate reductase, proteolytic, lipolytic, Amylolytic activities and antifungal activity. A series of *in vitro* fermentation experiments were performed with varying concentrations of NaCl, $NaNO_2$, glycerol and meat fat particles. A low concentration of NaCl (2%, w/v) showed almost no effect on bacterial growth. However, the biomass concentration in the presence of 4 and 6% NaCl was 91 and 72% of the concentration which was obtained when no salt was added. All sodium nitrite concentrations tested (100, 200 and 300 ppm) had little or no influence on both growth and bacteriocin production by *E. faecium* SFD with the exception of 400 ppm $NaNO_2$. Furthermore, the strain was able to reduce nitrate to nitrite at anaerobic conditions and showed proteolytic activities as revealed by clear haloes in milk agar. Also an antifungal activity was detected against *Fusarium proliferatum*, *F. culmorum*, *F. moniliforme* and *F. oxysporum*. The restrictive and inhibitory effects of nitrite, salt and fat on the growth of bacteria investigated were not very marked, and there were no significant differences between the separate or combined action of salt and nitrite.
[Sahar F. Deraz, Mohammed G. Shehata, Ashraf A. Khalil. **Significant Industrial Properties of *Enterococcus faecium* SFD as a Probiotic and Bacteriocin-Producing Strain.** *Life Sci J* 2015;12(3):82-91]. (ISSN:1097-8135). http://www.lifesciencesite.com. 11

Keywords: lactic acid bacteria; *E. faecium*; cell biomass; sodium chloride; sodium nitrite**;** antifungal

1. Introduction

Food safety remains a major public health concern and challenge to food producer to provide adequate protection to consumers. On the other hand, consumers want food with less or without chemical additives which could lead to a potential loss of protection from processing. Consumers increasingly prefer more stable, safer and natural food products that are mild and light with low acid, sugar, salt, and fat content (Gould 1996). With the surging interest for natural preservatives and biological preservation techniques, the application of lactic acid bacteria (LAB) as starter or protective cultures has attracted great interest as safe food grad preservatives of biological origin (Holzapfel et al., 1995). LAB are generally recognized as safe (GRAS microorganisms) and their antimicrobial potential play an important role in the preservation, microbiological stability and development of starter cultures for fermented foods (O'Sullivan et al., 2002). The preservative effect exerted by LAB is mainly due to acid production, competition for nutrients, formation of hydrogen peroxide, CO_2, diacetyl D-isomers of amino acids, reuterin or antimicrobial peptides, i.e. bacteriocins (Klaenhammer 1988; Cintas et al., 2001). This potential property of LAB allows biological control of foodborne pathogens without resorting to more severe physical treatments (Cleveland et al., 2001). Since the isolation and screening of microorganisms from natural sources has always been the most powerful means for obtaining useful and genetically stable strains for industrially important products (Ibourahema et al., 2012). The strain tested in the current study was isolated and identified as bacteriocinogenic LAB from Egyptian karish cheese, then evaluated their antimicrobial effects *in vitro* against *Salmonella enterica*, *Bacillus cereus* and *Yersinia enterocolitica* with strong activity of 25600 AU/mL against *Salmonella enterica* and *Bacillus cereus*. The newly isolated culture was further evaluated for a number of probiotic characteristics like viability under gut-like conditions, cell surface hydrophobicity, autoaggregation, coaggregation, Phenol tolerance, adhesion to intestinal mucousand cholesterol assimilation. *In vitro* results obtained showed that the isolated *Enterococcus faecium* SFD, was able to meet the basic requirements for probiotic functions as they demonstrated probiotic characteristics such as tolerance to pH 2.5, growth in 0.5% bile salts, growth in 0.3% phenol, hydrophobicity of 37%, adhesion to intestinal mucous and 35% cholesterol assimilation ability. In the light of this study, it was observed that, *E. faecium*

SFD is regarded as a promising candidate probiotic and adjunct culture for new healthy food product (Deraz et al., 2013). The enterococci are lactic acid bacteria (LAB) that are important in environmental and food microbiology. Enterococci often occur in large numbers foods, especially those of animal origin such as dairy products (Giraffa, 2003) and considered as a part of the natural intestinal flora of humans and animals and play an important role in maintaining the microbial balance (Leroy and Vuyst, 2002). These bacteria play a beneficial role in the development of the sensory and quality characteristics of fermented foods and have been successfully used in many different applications as starter or adjunct cultures, and as probiotics (Centeno et al., 1996; Wessels et al., 1990). The effect and different applications of enterococci in food and health and more specifically in meat and dairy products have been reviewed (Giraffa, 2003; Foulquie´ Moreno et al., 2006; Hugas, 1998). Enterococci possess a competitive advantage over other microbiota in meat fermentations, and many enterococci isolated from sausages have the ability to produce enterocins harbouring antimicrobial activity against pathogens and spoilage microorganisms of meat concern (Hugas, 1998). The manufacture of fermented sausages includes mixing of minced meat and fat with salts, curing agents and spices, stuffing into casings and fermentation of the mixture by a well-defined starter culture under controlled conditions. Adding salt (2.5 to 3.0%, wt/wt) to raw sausage is essential; salt decreases water activity (a_w) and contributes to flavor and microbial selection (Leistner, 1995). Adding nitrate and nitrite to sausage batter is common. Nitrite is added to produce color, to prevent lipid rancidity, and to inhibit the growth of Salmonellae and Clostridia (Leistner, 1995; Drosinos, 2007), and to obtain the typical cured flavor (Dainty and Blom, 1995). The concentration of sodium nitrite may vary from 20 to 200 ppm depending on the type of sausage and on the legislation (Krockel 1995; Deraz et al., 2009). In this study, the effects of typical sausage ingredients and other technological factors on the functionality of *E. faecium* SFD in the meat environment were investigated.

2. Material and Methods

Bacterial strains and media

Enterococcus faecium SFD was used as the producer of the antipathogenic bacteriocin (Deraz et al., 2013). A sensitive indicator organism, *Salmonella enterica* ATCC 25566 was used to determine bacteriocin activity levels. Strain of *E. faecium* SFD was isolated from Egyptian kareish cheese and was routinely growth in MRS agar (Oxoid, Milan, Italy) at 37ºC for 18–24 hr. Strains were stored at -80°C in MRS medium containing 25% (v/v) glycerol as a cryoprotectant. To produce fresh cultures, the bacteriocin producer strain *E. faeicum* SFD and the indicator strain *Salmonella enterica* ATCC 25566 were propagated twice in MRS and nutrient broth at 37°C for 16-18 hr before experimental use, respectively. Solid medium was prepared by adding 1.5% (w/v) agar (Difco Laboratories) to the broth. The overlays used for estimation of bacteriocin titers were prepared with 0.75% (w/v) agar.

Fermentation experiments

A series of *in vitro* fermentation experiments were carried out in MRS broth inoculated with 1% (v/v) of freshly prepared *E. faeicum* SFD and incubated under agitation (80 rpm) at 37°C. These fermentations were performed using fermentation liquors with added NaCl and $NaNO_2$ at various concentrations (2, 4 and 6% [w/v] and 100, 200, and 400 ppm [w/v], respectively, (the latter was sterilized separately by microfiltration [Sartolab; Sartorius, Germany]). An additional fermentation experiment was performed without salt and nitrite. The fermentations containing 0 or 4% (w/v) NaCl were performed in triplicate so as to determine reproducibility of the experiments.

To determine whether the effect of the added NaCl was due solely to a reduction in a_w, an additional fermentation was carried out in the presence of 9.9 and 21.1% (w/v) glycerol (sterilized separately) in the absence of salt. Based on extrapolation of data from previous published studies, addition of 9.9 and 21.1% (w/v) glycerol to the basal growth medium should result in an a_w similar to 4 and 8% (w/v) NaCl, respectively (Chandler and McMeekin, 1989; McMeekin et al., 1987).

In another set of fermentation experiments, a modified MRS (mMRS) was used as the fermentation medium for *E. faeicum* SFD. The concentration of the complex nutrient sources i.e. bacteriological peptone (Oxoid), meat extract (Oxoid), and yeast extract (VWR International) was doubled. This modification to the standard MRS medium was done to investigate potential growth limitation of *E. faeicum* SFD due to nutrient depletion. In addition, calculations of the amino nitrogen content of MRS medium (Bridson, 1998) indicated that this composition more closely simulates the actual sausage environment (Dainty and Blom, 1995). All media and solutions were sterilized at 121°C for 20 min.

Assays

At regular time intervals, samples were withdrawn aseptically to determine biomass (optical density at 600 nm) and the level of soluble bacteriocin activity in a cell-free culture supernatant. Bacterial growth was performed with biomass concentrations obtained from OD 600 measurements to allow comparison with results obtained previously with other

strains (Holck et al., 1992) as well as with other data available from literature.

Bacteriocin activity assay

The culture supernatants were assayed for bacteriocin activity by the spot on lawn technique with MRS agar using *Salmonella enterica* ATCC 25566 as indicator strain (Barefoot and Klaenhammer, 1983; Deraz et al., 2005). Indicator lawns were prepared by adding 0.125 mL of 10 times diluted overnight culture to 5 mL of MRS soft agar (0.75%). The contents of the tubes were gently mixed and poured over the surfaces of pre-poured MRS agar plates. Bacteriocin samples were sterilized by passage through a 0.22 µM cellulose acetate filter. Serial two-fold dilutions were carried out in the same medium as used for the growth of the indicator strain. Activity was quantified by taking the reciprocal of the highest dilution that exhibited a clear zone of inhibition and was expressed as activity units (AU) per milliliter of culture media. The titre of the bacteriocin solution, in AU/mL, was calculated as (1000/d) D, where D is the dilution factor and'd' is the dose (the amount of bacteriocin solution pipetted on each spot) (Deraz et al., 2005). To avoid errors in bacteriocin activity values, the same person made all observations. Moreover, it was checked to confirm that salt and nitrite did not interfere with the bacteriocin activity assay method.

Adsorption of bacteriocin to fat and meat

Bacteriocin-containing cell-free culture supernatant was prepared by inoculating 1 L of MRS broth (Oxoid) with *E. faecium* SFD (1%, v/v), incubation overnight at 37˚C and removing the cells by centrifugation ($6{,}500_{xg}$, 20 min). Luncheon meat composed of beef meat, salt, spices, 0.05% ascorbic acid and 100 ppm sodium nitrite with low fat content, no more than 22%, (the entire product) or beef fat (further referred to as fat) were obtained from a local store and flamed to reduce the superficial contamination. Fat or luncheon particles (100 or 300g l^{-1}, ± 2g) were added to the supernatant (50 mL) and stored at refrigerator temperatures. Bacteriocin activity in the supernatant was measured over time. A control experiment was performed in the absence of meat and fat.

Proteolytic activity

Surface-dried plates of milk agar (Gordon et al., 1973) were streaked with 24-h-old cultures, after incubation at 30 ºC for 4 days, and examined for any clearing of casein around and underneath the growth for assessment of proteolytic activity.

Amylolytic activity

Surface-dried plates of starch agar (Gordon et al., 1973) were streaked with 24-hr-old cultures, after incubation at 30°C for 4 days. The plates were flooded with iodine solution for 15–30 min and examined the clear zone underneath (after the growth was scrapped off) for amylolytic activity.

Lipolytic activity

Surface-dried plates of tributyrin agar (Leuschner et al., 1997) were streaked with 24-hr-old culture, after incubation at 30°C for 4 days. Lipolytic activity was detected by a clear zone surrounding the culture in the turbid tributyrin agar.

Screening for antifungal potential

The antifungal activity of the LAB strain was investigated with an overlay assay (Lind et al., 2005; Maganusson and Schnurer, 2001). Micro-organisms used for the antifungal activity are *Fusarium proliferatum*, *F. culmorum*, *F. moniliforme* and *F. oxysporum*. The antifungal activity was evaluated by measuring the inhibition zone of the fungal growth and the scale was the following:

- = no inhibition zone; + = inhibition zone of less than 6 mm; ++ = inhibition zone of 6 – 10 mm;

+++ = inhibition zone of 10 – 18 mm; ++++ = inhibition zone of more than 20 mm.

Nitrate reductase activity

Nitrate reductase activity was determined as described by Miralles et al. 1996 on MRS supplemented with 1 g l^{-1} KNO_3. The cell pellet of an overnight culture was resuspended in equal volume of 50 mM phosphate buffer pH 7.0 and 30 µL loaded into agar plates wells (6 mm diameter). After incubation at 30ºC for 12 to 14 hr the plates were flooded with 1 mL of a 1:1 solution of NIT1 (0.8 g sulphanilic acid in 100 mL of acetic acid 5 N) and NIT2 (0.6 g N-N-dimethyl-1-Naphthylamine in 100 mL of acetic acid 5 N) for the detection of nitrite. The appearance of red haloes surrounding the wells indicated the presence of nitrate reductase activity.

3. Results and Discussion

Effect of NaCl

Tolerance to NaCl is a significant feature for starter culture to compete with the natural microbiota of the raw material and to undertake the metabolic activities expected in the conditions prevailing in the sausage (Ammor and Mayo, 2007). According to Olesen et al., 2004, the initial salt concentration in the sausage is about 2 to 3% and can reach 4.2-6.0% in the final product due to the loss of moisture (Moretti et al., 2004; Zanardi et al., 2004; Papadima et al., 1999; Sawitzki et al., 2009). In the case of *E. faecium* SFD, addition of 2, 4 and 6% (w/v) NaCl gave similar profile of biomass (Figure 1). A low concentration of NaCl (2%, w/v) showed almost no effect on bacterial growth, while an inhibition that increased linearly was evident with higher salt concentrations. The biomass concentration in the presence of 4 and 6% NaCl was 91 and 72% of the concentration which was obtained when no salt was added. The negative effect of high salt concentrations on the growth of LAB has been

reported previously (De Vuyst et al., 1996; Roze´s and Peres 1996; Uguen et al., 1999). However, low concentrations of salt (1 to 2%, w/v) can sometimes enhance bacterial growth (Uguen et al., 1999; Ganzle et al., 1998; Vignolo et al., 1995; Korkeala et al., 1992). Salt tolerance may be due to its ability to efficiently accumulate osmo- and cryoprotective solutes such as betaine and carnitine (Ammor and Mayo, 2007).

Effect of $NaNO_2$

The addition of sodium nitrite to the production medium had a mixed effect on the on the growth rate and bacteriocinogenicity of the strain. The radii of growth inhibition zones and growth rat associated with *E. faceium* SFD decreased with increasing salt concentrations and markedly affected by 400 ppm in the production medium (Figure 2). Nitrite is known mainly for its antimicrobial activity against spore formers; it has a limited effect on the growth of lactic acid bacteria at concentrations less than 200 mg liter $^{-1}$ (Vignolo et al., 1995; Korkeala et al., 1992; Doßmann et al., 1996), but at 400 mg liter^{-1} inhibition is more pronounced (Korkeala et al., 1992). Nitrite 80 to 240 (in U.S. 156) mg/kg is used for antibacterial, colour, and antioxidant purposes. Initial nitrite level is ~100 ppm; however, as little as 50 ppm improves flavour and appearance, 30-50 ppm is sufficient to produce cured colour, and 100 ppm will produce a desirable flavour and appearance (Lueck and Hechelmann, 1987).

During the fermentations carried out in mMRS medium without and with 100 ppm of added $NaNO_2$ under standard conditions, *E. faecium* SFD grew exponentially for approximately 10 to 12 hr, after which growth slowed down (Figure 2). After 16 to 18 hr of fermentation, growth completely ceased. In contrast, for *Enterococcus faecium* CTC 492, the addition of 100 ppm of $NaNO_2$ significantly inhibited enterocin production compared with that seen in the standard MRS control (Aymerich et al., 2000). The addition of 200 and 400 ppm $NaNO_2$ caused the bacteria to enter into the stationary phase later after 18 hr, with reducing biomass production.

All sodium nitrite concentrations tested had little or no influence on bacteriocin production by *E. faecium* SFD with the exception of 400 ppm $NaNO_2$ (Figure 2). When 400 ppm $NaNO_2$ was added, no bacteriocin activity could be detected before 14 hr of fermentation time and reached the maximum after 22 hr. This decrease in bacteriocin activity was due to a marked inhibition of biomass production. Different explanations have been suggested that the presence of un-dissociated nitrous acid molecules, enhancing the toxic effect of lactic acid, as a result of intracellular accumulation (Leroy and De Vuyst 1999a and b). Furthermore, it has been mentioned previously that nitrite might interfere with active transport mechanisms (Davidson, 1997), which could explain the surprisingly low biomass obtained when 0.04% sodium nitrite is used. However, in general *E. faecium* SFD could produce significantly large amounts of bacteriocin activity (25600 arbitrary units/mL) in the presence of different concentrations of $NaNO_2$ which clearly, showed distinct adaptive responses to environmental stress conditions.

Combined effect of NaCl and $NaNO_2$

The combined action of salt and nitrite influenced both the growth rate and bacteriocin production of *E. faecium* SFD to similar extent. The fermentation carried out in the presence of 2% NaCl and 100 ppm nitrite results in biomass concentration comparable to the fermentation without NaCl or Nitrite, except for a slightly increase in the lag phase (from 12 to 14hr) compared to the reference fermentation in which no NaCl or Nitrite was added. The combined action of 2% NaCl and 100 ppm $NaNO_2$ led to recovery of 94 and 100% of cell biomass and bacteriocin production of the reference fermentation after 16 hr (Figure 3) which was similar the separate effect of either 2% NaCl or 100 ppm $NaNO_2$. However, addition of the same concentration of NaCl (2%) and double concentration of sodium nitrite (200 ppm) or the exact concentration of sodium nitrite (100ppm) and double concentration of NaCl (4%) led to drastic change by slowing down of both biomass and bacteriocin production. The maximum of both bacteriocin production and cell biomass was stimulated after 20 and 18 hr of fermentation, respectively. The higher bacteriocin activity obtained in the presence of different concentrations of both sodium chloride and sodium nitrite was the consequence of attainable biomass concentration comparable to the maximum attainable biomass concentration of the reference fermentation. However, the obtained activity probably is sufficient to have a significant antibacterial effect in the sausage environment, as demonstrated by Hugas et al., 1995.

Influence of a_w

Glycerol considered as one of the agent which decreased the a_w. Therefore, to determine whether a reduction in a_w was solely responsible for the inhibitory effect of NaCl on bacterial growth, or other (ionic) effects were responsible, two fermentation experiments were performed with glycerol added as an a_w–lowering agent. The presence of glycerol in the growth medium had similar or stronger effects on the growth rate as was the case for salt. In this study, the biomass values obtained for fermentation in the presence of 9.9% (99 g 1^{-1}) glycerol were roughly comparable to the putative effects of 4.0% (60 g 1^{-1}) sodium chloride. However, at higher glycerol concentration of 21.1% (211 g 1^{-1}) a stronger decrease in the growth rate and biomass values was seen which was higher than the effect of 6% NaCl. In general, biomass production was lower when glycerol was used as a_w–lowering agent instead of

NaCl (Figure 4). Similar results have been reported previously (Chandler and McMeekin, 1989; McMeekin et al., 1987), in which addition of 9.9 and 21.1% glycerol to the basal growth medium have resulted in the same decreases in the a_w as approximately 4 and 8% sodium chloride, respectively.

Adsorption of bacteriocin to fat and meat particles

The bioavailability of bacteriocin in the food ecosystem can severely affect by some food limiting factor including, adsorption of the bacteriocin onto meat particles, fats, and proteins. In this work, the relationship between bacteriocin activity produced by *E. faecium* SFD and fat content was assessed. The level of bacteriocin activity remained practically constant for 10 to 14 hr depending on the fat content. However, measurable bacteriocin activity disappeared from the bacteriocin-containing culture supernatant (Figure 5) after 16 and 14 hr in the presence of 100 and 300 g beef fat, respectively. The depletion of bacteriocin in culture supernatant was dependent on the amount of fat added, with the apparent inactivation being higher at 300 than at 100 g fat l^{-1}.

On the other hand, bacteriocin activity in culture supernatant with luncheon meat particles was rather less stable comparable to bacteriocin activity in the presence of meat fat which can be referred to the presence of other Interfering food constituents include high salt concentration (Boziaris and Nychas, 2006), nitrates (Ghalfi et al., 2006). However, as it appears in Figure 5 a significant level of bacteriocin activity can be detected up to 14 to 20 hr of incubation time based on the luncheon content. Similar observation has been reported for nisin (Ghalfi et al., 2006), Pentocin 31-1 (Guo-rong et al., 2012; Zhang et al., 2010) and sakacin P (Urso et al., 2006).

These results showed that the complex meat constituents may provoke the in situ action of bacteriocins. One of the most common features of bacteriocins from lactic acid bacteria is its hydrophobic nature which is likely to enhance unspecific binding of the bacteriocin to the hydrophobic surfaces of fat particles (Holzapfel et al., 1995). However, this hydrophobic nature does not necessarily led to loose of its antibacterial activity and still have its surface action against potential spoilers or pathogens (Guo-rong et al., 2012).

Proteolytic, lipolytic and amylotic activity

The strain of *E. faecium* SFD isolated from the Egyptian Karash cheese showed proteolytic activities as revealed by clear haloes in milk agar (showing >2mm hydrolysis zone in milk agar plate) (Figure 6). Our result was however not a surprise since many strains of LAB isolated from cheese have been reported to produce high proteolytic activity (Durlu–Ozkaya et al., 2001; Dagdemir and Ozdemir). The proteolytic activity of dairy lactic acid bacteria is essential for their growth in milk leading to the development of required organoleptic properties of different fermented milk products (Christensen et al., 1999; Hassaine et al., 2007). The proteolytic activity and acid production of enterococci during cultivation in milk are to some extent comparable to those of *Streptococcus thermophiles* (Gatti et al., 1994). However, the strain was unable to hydrolyze either tributyrin or starch showing no lipolytic and amylotic activity.

This result was in contrast with those of Thapa et al. (2006), who reported that *Enterococcus feacium* isolated from fish was lipolytic. Amylolytic lactic acid bacteria produce amylases to facilitate hydrolysis and fermentation of starch to lactic acid (Reddy et al., 2008). No amylase producing strain of LAB has been reported from dairy products. However, most amylase producing strains have been reported in LAB isolated from cereal fermented products (Olasupo et al., 1996; Agati et al., 1998; Sanni et al., 2002; Omafuvbe and Enyioha 2011).

Antifungal activity

The antifungal activity of *E. faecium* SFD against *Fusarium proliferatum*, *F. culmorum*, *F. moniliforme*, *F. oxysporum* and *Aspergillus niger* was measured and the results are shown in Table 1. Varying degrees of inhibition was detected against the different strains of *Fusarium* with inhibition zones ranged from 11.5 to 22.5 mm. However, no activity against *Aspergillus niger* has been detected. The inhibition zone was reduced when the culture supernatants were used (data not shown), which could have been due to a low concentration of antifungal compounds. The detected antifongic activity against different *Fusarium* species can be due to many antifungal compounds produced by lactic acid bacteria including, organic acids, hydrogen peroxide, reuterin, proteinaceous compounds and cyclic dipeptides (Schnurer and Magnusson 2005). The lactic acid bacterial activities are extensively studied while less efforts has been paid to explore its antifungal activity (Rehaiem et al., 2010; Valerio et al., 2009). In previous study, the bacteriocin production by *E. faecium* SFD has been confirmed against both gram positive and negative bacteria including many spoilage and pathogenic bacteria such as, *Salmonella enterica*, *Bacillus cereus* and *Yersinia enterocolitica* (Deraz et al., 2013). In agreement of the finding of our study, only a few studies cite a wide spectrum antifungal activity for isolates of lactic acid bacteria (Storm et al., 2002) . However, other studies were suggested a more strain specific antifungal activity against species of one or two major mold genera (Niku-Paavola et al., 1999; Florianowicz, 2001; Gourama and Bullerman, 1997; Sudun and Miyamoto 2001).

Nitrate reductase activit

In a long curing process, nitrate reductase activity is considered to be the most important property of the starter cultures for fermented sausages production (Weber, 1994). The reduction of nitrate to nitrite is essential to promote the reddening process of the cured meat as a result of the action of nitrate reductase enzymes which normally derives from Micrococcacea (Marco et al., 2006; Buchenhuskes 1993). In this study the tested *E. faecium* SFD showed nitrate reductase activity as confirmed by the appearance of red haloes surrounding the wells. According to Buchenhuskes (1993), the curing of meat products in the absence of cocci is only accepted when the lactic acid bacteria present have significant high nitrate reductase activities. Therefore, if the product fermentation process demands activity of nitrate reductase, the use of the *E. faecium* SFD strain isolated could be used to exhibit the respective activity. Bacteriocin-producing strains may be used to replace part of the preservative action, whereas the use of strains that are able to reduce nitrate to nitrite, could be useful to partially take over the colouration function of the curing agents. The latter possibility has been demonstrated with strains of *Lactobacillus fermentum* (Xu and Verstraete 2001; Moller et al., 2003).

Table 1. Antifungal activity of *E. faecium* SFD against bread spoilage moulds.

Fungi	Inhibition Zone
F. proliferatum	+++
F. culmorum	++++
F. moniliforme	++++
F. oxysporum	+++
Aspergillus niger	-

- = no inhibition zone
+ = inhibition zone of less than 6 mm
++ = inhibition zone of 6 – 10 mm
+++ = inhibition zone of 10 – 18 mm
++++ = inhibition zone of more than 20 mm

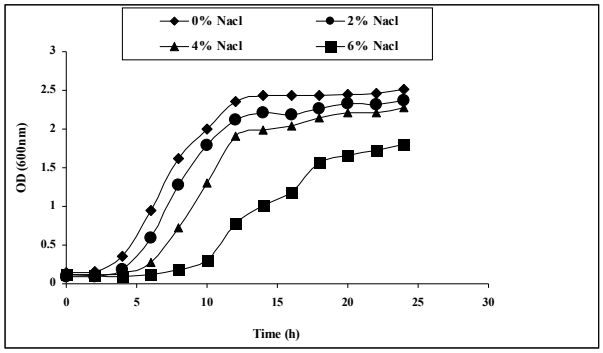

Figure 1. Influence of different concentrations of NaCl on the growth of *E. faecium* SFD, as measured by optical density at 600 nm (OD600) as a function of time. NaCl was added at 20 g $^{l-1}$ (2%), 40 g $^{l-1}$ (4%), 60 g $^{l-1}$ (6%)

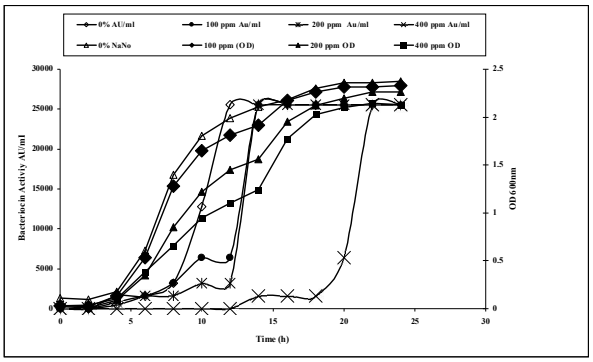

Figure 2. Influence of different concentrations of sodium nitrite on the growth of *E. faecium* SFD, as measured by optical density at 600 nm (OD600) and bacteriocin activity as a function of time. Sodium nitrite was added at 0, 100, 200 and 400 ppm.

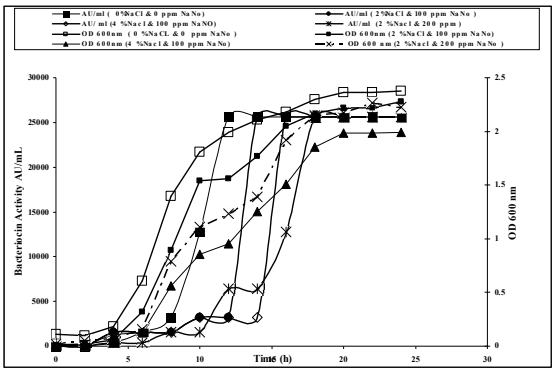

Figure 3. Influence of different concentrations of sodium chloride combined with different concentration of sodium nitrite on the growth of *E. faecium* SFD, as measured by optical density at 600 nm (OD600) and bacteriocin activity as a function of time. Sodium chloride and sodium nitrite was added at 0g l-1 (0%) + 0ppm, 20g l-1 (2%) +100 ppm, 20g l-1 (2%) + 200 ppm and 40g l-1 (4%) + 100 ppm, respectively.

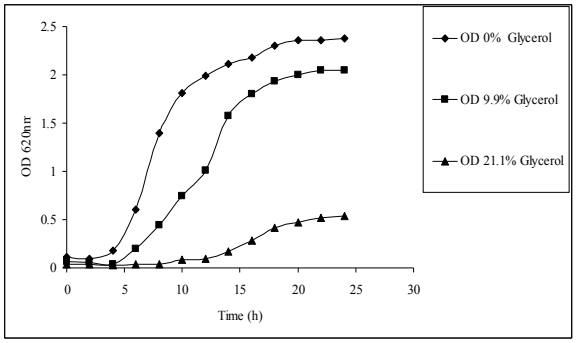

Figure 4. Influence of different concentrations of glycerol on the growth of *E. faecium* SFD, as measured by optical density at 600 nm and bacteriocin activity as a function of time. Glycerol was added at 9.9 and 21.1%

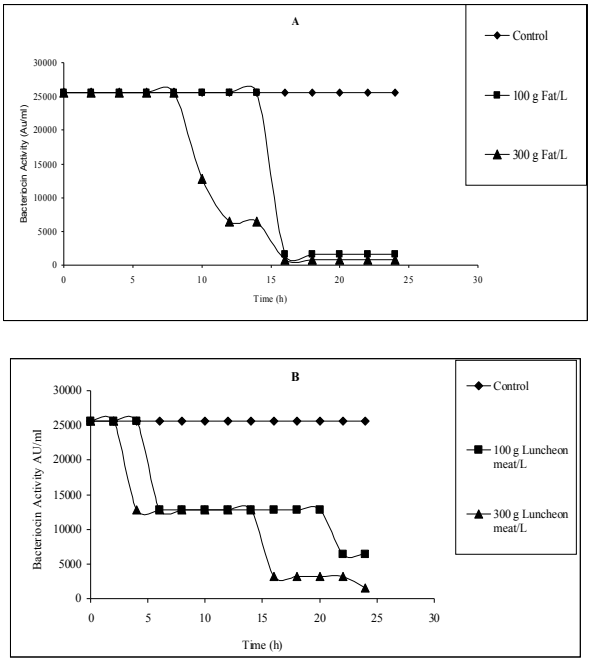

Figure 5. Bacteriocin activity in MRS culture supernatant in the presence of 100g (■) or 300g (▲) of fat l^{-1} (A) and in the presence of 100g (■) or 300g (▲) of luncheon meat l^{-1}. (♦) symbol represents control experiments in the absence of fat (A) or luncheon meat (B).

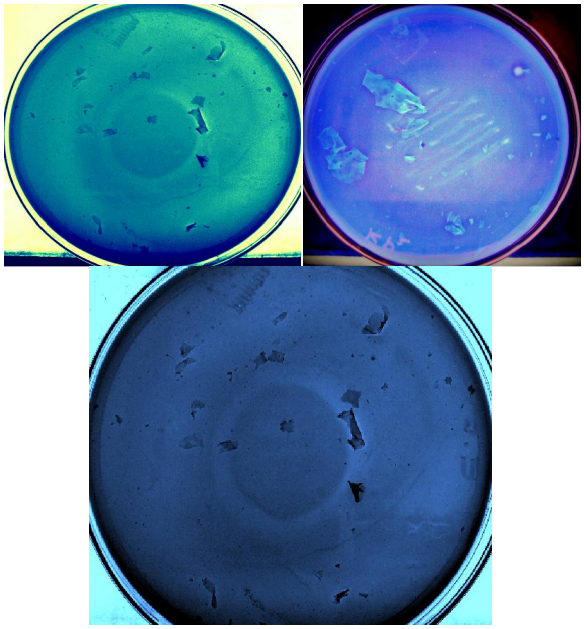

Figure 6. Proteolytic activity of *E. faecium* SFD strain grown on skim milk plat, the clear zone indicate the presence of proteolytic activity

4. Conclusion

Although all experiments were conducted in a model system, these findings will aid in industrial implementation of efficient bacteriocin-producing protective cultures of *E. faecium* SFD in meat production. These bacteriocinogenic protective cultures will contribute to meat products that are safer and of more consistent quality.

Running title: Significant Industrial Properties of *Enterococcus faecium* SFD

References

1. Gould GW. Industry perspectives on the use of natural antimicrobials and inhibitors for food applications. J Food Prot 1996;Suppl.:S82–S6.
2. Holzapfel WH, Geisen R, Schillinger U. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. Int J Food Microbiol 1995;24: 343-62.
3. O'Sullivan L, Ross RP, Hill C. Potential of bacteriocin producing lactic acid bacteria for improvements in food safety and quality. Biochimie 2002;84(5/6):593-604.
4. Klaenhammer TR. Bacteriocins of lactic acid bacteria. Biochimie 1988;70(3):337-49.
5. Cintas LM, Casaus MP, Herranz C, Nes IF, Hernández PE. Bacteriocins of lactic acid bacteria. Food Sci Tech Int 2001;7:281–305.
6. Cleveland J, Montville TJ, Nes IF, Chikindas ML. Bacteriocins: safe, natural antimicrobials for food preservation. Int J Food Microbiol 2001;71(1):1-20.
7. Ibourahema C, Dauphin RD, Jacqueline D, Thonart P. Characterization of lactic acid bacteria isolated from poultry farms in Senegal. African J Biotechnol 2008:7:2006–12.
8. Deraz SF, Shehata MG, El-Banna AA, Khalil AA, El-Sahn MA. A complementary ''*In Vitro*'' study of bacteriocinogenic activity and probiotic characteristics of newly isolated *Enterococcus faecium* SFD. J Pure Appl Microbiol 2013;7(4):2673-89.
9. Giraffa G. Functionality of enterococci in dairy products. Int J Food Microbiol 2003;88: 215–22.
10. Leroy F, De Vuyst L. Bacteriocin production by *Enterococcus faecium* RZS C5 is cell density limited and occurs in the very early growth phase. Int. J Food Microbiol 2002;72: 155–64.
11. Centeno JA, Menendez S, Rodriguez-Otero JL. Main microbial flora present in natural starters in Cebreiro raw cow's milk cheese, Northwest Spain. Int J Food Microbiol 1996;33:307–13.
12. Wessels D, Jooste PJ, Mostert JF. Technologically important characteristics of

Enterococcus isolates from milk and dairy products. Int J Food Microbiol 1990;10:349–52.
13. Foulquie´ Moreno MR, Sarantinopoulos P, Tsakalidou E, De Vuyst L. The role and application of enterococci in food and health. Int J Food Microbiol 2006;106:1–24.
14. Hugas M. Bacteriocinogenic lactic acid bacteria for the biopreservation of meat and meat products. Meat Sci 1998;49:S139–S50.
15. Leistner L. Stable and safe fermented sausages world-wide. In Fermented Meats, Campbell-Platt G., Cook PE. Eds.; Blackie Academic & Professional, London, United Kingdom, 1995; p.160– 75.
16. Drosinos EH, Paramithiotis S, Kolovos G, Tsikouras I, Metaxopoulos I. Phenotypic and technological diversity of lactic acid bacteria and staphylococci isolated from traditionally fermented sausages in southern Greece. Food Microbiol 2007;24:260-70.
17. Dainty R, Blom H. Flavour chemistry of fermented sausages. In Fermented Meats, Campbell-Platt G., Cook PE. Eds.; Blackie Academic & Professional, London, United Kingdom,1995; p.176– 193.
18. Krockel L. Bacterial fermentation of meats. In Fermented Meats, Campbell-Platt G., Cook PE. Eds.; Blackie Academic & Professional, London, United Kingdom, 1995; p.69– 109.
19. Deraz SF, Khalil AA, Abd-Ellatif SA, Ghreeb DA, El-Dewany AI. Effects of curing agents on growth and Sakacin A production by *Lactobacillus sakei* Lb 706 during production of processed meat. J Appl Biosci 2009;16:845–56.
20. Chandler RE, McMeekin TA. Modelling the growth response of *Staphylococcus xylosus* to changes in temperature and glycerol concentration/ water activity. J Appl Bacteriol 1989; 66:543–48.
21. McMeekin TA, Chandler RE, Doe PE, Garland CD, Olley J, Putro S, Ratkowsky DA. Model for combined effect of temperature and salt concentration/water activity on the growth rate of *Staphylococcus xylosus*. J Appl Bacteriol 1987;62:543–50.
22. Bridson EY. The Oxoid manual. 8th ed. Oxoid, Basingstoke, United Kingdom,1998.
23. Holck A, Axelsson L, Birkeland S, Aukrust T, Blom H. Purification and amino acid sequence of sakacin A, a bacteriocin from *Lactobacillus sake* Lb 706. J General Microbiol 1992;138:2715-20.
24. Barefoot SF, Klaenhammer TR. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl Environ Microbiol 1983;45:1808-15.
25. Deraz S, Karlsson NE, Hedström M, Andersson MM, Mattiasson B. Purification and characterisation of acidocin D20079, a bacteriocin produced by *Lactobacillus acidophilus* DSM20079. J Biotechnol 2005;117:343-54.
26. Gordon RE, Haynes WC, Pang CHN. The Genus *Bacillus*, Hand-book no.427. United States Department of Agriculture, Washington, DC, 1973.
27. Leuschner RG, Kenneally PM, Arendt EK. Method for the rapid quantitative detection of lipolytic activity among food fermenting microorganisms. Int J Food Microbiol 1997;37: 237-40.
28. Lind H, Jonsson H, Schnurer J. Antifungal effect of dairy propionibacteria contribution of organic acids. Int J Food Microbiol 2005;98:157-65.
29. Maganusson J, Schnurer J. *Lactobacillus coryniformis* subsp. *Coryniformis* strain Si3 produces a broad-spectrum proteinaceous antifungal compound. Appl Environ Microbiol 2001;67:1-5.
30. Miralles MC, Flores J, Perez-Martinez G. Biochemical tests for the selection of *Staphylococcus* strains as potential meat starter cultures. Food Microbiol 1996;13:227–36.
31. Ammor MS, Mayo B. Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. Meat Sci 2007;76:138-46.
32. Olesen PT, Meyer AS, Stahnke L. Generation of flavour compounds in fermented sausages – the influence of curing ingredients, Staphylococcus starter culture and ripening time. Meat Sci 2004;66:675-87.
33. Moretti VM, Madonia G, Diaferia C, Mentasti T, Paleari MA, Panseri S, Pirone G, Gandini G. Chemical and microbiological parameters and sensory attributes of a typical Sicilian salami ripened in different conditions. Meat sci 2004;66:845-54.
34. Zanardi M, Ghidini S, Battaglia A, Chizzolini R. Lipolysis and 587 lipid oxidation in fermented sausages depending on different processing conditions and 588 different antioxidants. Meat Sci 2004;66:415-23.
35. Papadima SN, Arvanitoyannis IS, Bloukas JG. Chemometric model for describing Greek traditional sausages. Meat sci 1999;51:271-77.
36. Sawitzki MC, Fiorentini AM, Bertol TM, Sant'Anna ES. *Lactobacillus plantarum* strains isolated from naturally fermented sausages and their technological properties for application as starter cultures. Ciênc Tecnol Aliment. Campinas 2009;29(2):340-45.

37. De Vuyst L, Callewaert R, Crabbe K. Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. Microbiol 1996;42:817–27.
38. Roze´s N, Peres C. Effect of oleuropein and sodium chloride on viability and metabolism of *Lactobacillus plantarum.* Appl Microbiol Biotechnol 1996;45:839–43.
39. Uguen P, Hamelin J, Le Pennec JP, Blanco C. Influence of osmolarity and the presence of an osmoprotectant on *Lactococcus lactis* growth and bacteriocin production. Appl Environ Microbiol 1999;65:291–3.
40. Ganzle MG, Ehmann M, Hammes WP. Modeling of growth of *Lactobacillus sanfranciscensis* and *Candida milleri* in response to process parameters of sourdough fermentation. Appl Environ Microbiol 1998;64:2616–23.
41. Vignolo GM, de Kairuz MN, de Ruiz Holgado AAP, Oliver G. Influence of growth conditions on the production of lactocin 705, a bacteriocin produced by *Lactobacillus casei* CRL 705. J Appl Bacteriol 1995;78:5–10.
42. Korkeala H, Alanko T, Tiusanen, T. Effect of sodium nitrite and sodium chloride on growth of lactic acid bacteria. Acta Vet Scand 1992;33:27–32.
43. Doßmann MU, Vogel RF, Hammes WP. Mathematical description of the growth of *Lactobacillus sake* and *Lactobacillus pentosus* under conditions prevailing in fermented sausages. Appl Microbiol Biotechnol 1996;46:334–9.
44. Lueck F, Hechelmann H. Starter cultures for dry sausage and raw ham. Fleischwirtsch 1987; 67:307-14.
45. Aymerich MT, Artigas MG, Garriga M, Montfort JM, Hugas M. Effect of sausage ingredients and additives on the production of enterocins A and B by *Enterococcus faecium* CTC 492. Optimization of *in vitro* production and anti-listerial effect in dry fermented sausages. J Appl Microbiol 2000;88:686–94.
46. Leroy F, De Vuyst L. Temperature and pH conditions that prevail during fermentation of sausages are optimal for production of the antilisterial bacteriocin sakacin K. Appl Environ Microbiol 1999a;65:974–81.
47. Leroy F, De Vuyst L. The presence of salt and curing agent reduces bacteriocin production by *Lactobacillus sakei* CTC 494, a potential starter culture for sausage fermentation. Appl Environ Microbiol 1999b;65:5350–56.
48. Davidson PM. Chemical preservatives and natural antimicrobial compounds. In *Food microbiology: fundamentals and frontiers*, Doyle MP, Beuchat LR, Montville TJ. Eds, ASM Press, Washington, DC. 1997; p.520–56.
49. Hugas M, Garriga M, Aymerich MT, Monfort JM. Inhibition of *Listeria* in dry fermented sausages by the bacteriocinogenic *Lactobacillus sake* CTC 494. J Appl Bacteriol 1995;79: 322–30.
50. Boziaris IS, Nychas GJE. Effect of nisin on growth boundaries of *Listeria monocytogenes* Scott A, at various temperatures, pH, and water activities. Food Microbiol 2006;23:779-84.
51. Ghalfi H, Kouakou P, Duroy M, Daoudi A, Benkerroum N, Thonart P. Antilisterial Bacterioucin-producing Strain of *Lactobacillus curvatus* CWBI-B28 as a Preservative Culture in Bacon Meat and Influence of Fat and Nitrites on Bacteriocins Production and Activity. Food Sci Technol Int 2006;12(4):325-33.
52. Guo-rong L, Cheng-tao W, Yang W, Bao Z, Ping-lan L. Preservative effect of broad-spectrum enterocin LM-2 on cold sliced ham. Food Sci 2012;33(22):294-301.
53. Zhang J, Liu G, Li P, Qu Y. Pentocin 31-1, a novel meat-borne bacteriocin and its application as biopreservative in chill-stored traypackaged pork meat. Food Control 2010; 21(2):198-202.
54. Urso R, Rantsiou K, Cantoni C, Comi G, Cocolin L. Technological characterization of a bacteriocin-producing *Lactobacillus sakei* and its use in fermented sausages production. Int J Food Microbiol 2006;110(3):232-39.
55. Durlu–Ozkaya F, Xanthopoulos V, Tunail N, Litopoulous-Tzanetaki E. Technologically important properties of lactic acid bacteria isolates from Beyaz cheese made from raw ewe's milk. J Appl Microbiol 2001;91:861-70.
56. Dagdemir E, Ozdemir S. Technological characterization of natural lactic acid bacteria from artisanal Turkish white pickled cheese. Int J Dairy Technol 2008;61(2):133-40.
57. Christensen JE; Dudley EG, Pederson JA, Steele LJ. Peptidases and amino acid catabolism in lactic acid bacteria. Antonie Van Leeuwenhoek 1999;76:217-46.
58. Hassaine O, Zadi–Karam H, Karam N. Technological important properties of lactic acid bacteria isolated from raw milk of three breed of Algerian dromedary (Camelus dromedarius). Afr J Biotechnol 2007; 6(14):1720-27.
59. Gatti M, Fornasari E, Garni D, Giraffa G, Neviani E. Gli enterococchi nei formaggi italiani: attivita biochimiche e signifato technologica. Industria del Latte 1994;30:11-27.
60. Thapa N, Pal J, Tamang JP. Phenotypic identification and technological properties of lactic acid bacteria isolated from traditionally

processed fish products of the Himalayas. Int J Food Microbiol 2006;107:33-8.
61. Reddy G, Altaf M, Naveena BJ, Venkateshwar M, Kumar EV. Amylolytic 496 bacterial lactic acid fermentation — A review. Biotechnol Adv 2008;26:22-34.
62. Olasupo NA, Olukoya DK, Odunfa SA. Studies on local strains of Amylolytic Lactobaccillus from Nigerian fermented foods. Int J Food Res Dev 1996;40:45-46.
63. Agati V, Guyot JP, Morlon–Guyot J, Talamond P, Hounhouigan DJ. Isolation and characterization of newly amylolytic strains of *Lactobacillus fermentum* from fermented maize dough (Mawe and Ogi) from Benin. J Appl Microbiol 1998;85:512-20.
64. Sanni AI, Morlon–Guyot J, Guyot JP. New efficient amylase – producing starins of *Lactobacillus fermentum* isolated from different Nigerian traditional fermented foods. Int J Food Microbiol 2002;72:53-62.
65. Omafuvbe BO, Enyioha LC. Phenotypic identification and technological properties of lactic acid bacteria isolated from selected commercial Nigerian bottled yoghurt. Afr J Food Sci 2011;5(6):340– 48.
66. Schnurer J, Magnusson J. Antifungal lactic acid bacteria as biopreservatives. Trends Food Sci Tech 2005;16:70-8.
67. Rehaiem A, Martinez B, Manai M, Rodriguez A. Production of enterocin A by *Enterococcus faecium* MMRA isolated from Rayeb, a traditional Tunisian dairy beverage. J Appl Microbiol 2010;108:1685-93.
68. Valerio F, Favilla M, de Bellis P, Sisto A, de Candia S, Lavermicocca P. Antifungal activity of strains of lactic acid bacteria isolated from semolina ecosystem against *Penicillium roqueforti*, *Aspergillus niger* and *Endomyces fibuliger* contaminating bakery products. Syst Appl Microbiol 2009;32:438-48.
69. Storm K, Sjogren J, Broberg A, Schnurer J. *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo (L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. Appl Environ Microbiol 2002;68:4322-27.
70. Niku-Paavola MG, Laitila A, Mattial-Sandholm T, Haikara A. New types of antimicrobial compounds produced by *Lactobacillus plantarum*. J Appl Microbiol 1999;86:29-35.
71. Florianowicz T. Antifungal activity of some microorganisms against *Penicillium expansum*. Eur Food Res Technol 2001,212:282-86.
72. Gourama H, Bullerman LB. Anti-aflatoxigenic activity of *Lactobacillus casei pseudoplantarum*. Int J Food Microbiol 1997;34:131-43.
73. Sudun W, Miyamoto T. Screening and identification of lactic acid bacteria from Airag for antifungal activity. J Ani Vet Adv 2011;10:2751-57.
74. Weber H. Dry sausages manufacture, the importance of protective cultures and their metabolic products. Fleischwirtsch 1994;74:278–82.
75. Marco A, Navarro JL, Flores M. The influence of nitrite and nitrate on microbial, chemical and sensory parameters of slow dry fermented sausage. Meat Sci 2006;73:660-73.
76. Buchenhuskes HJ. Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities. FEMS Microbiol Rev 1993;12:253-72.
77. Xu J, Verstraete W. Evaluation of nitric oxide production by lactobacilli. Appl Microbiol Biotechnol 2001;56:504-7.
78. Moller JKS, Jensen JS, Skibsted LH, Knochel S. Microbial formation of nitrite-cured pigment, nitrosylmyoglobin, from metmyoglobin in model systems and smoked fermented sausages by *Lactobacillus fermentum* strains and a commercial starter culture. Eur Food Res Technol 2003;216:463–9.

2/26/2015