Short communication: Mycosporine-like amino acids protect natamycin against photodegradation in milk exposed to fluorescent or light-emitting diode light

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ABSTRACT

Natamycin has been used as a natural antimicrobial in dairy products, but the poor light stability of natamycin can limit usefulness in food products. Mycosporine-like amino acids are strong UV absorbers and might be useful as an additive to decrease light-induced degradations. Therefore, the objective of this study was to evaluate the photoprotective effect of mycosporine-like amino acids (MAA) in cow milk stored under 2 lighting conditions in retail commercial display cabinets with fluorescent and high intensity light-emitting diode illumination. When milk was exposed to both fluorescent and light-emitting diode light, natamycin degradation was very fast, and only $17.1 \pm 0.9\%$ of its original concentration was observed after 8 d at 4.0°C. On the other hand, by adding MAA into milk, natamycin was retained at $82.2 \pm 0.9\%$ and $92.2 \pm 0.9\%$ when low and high MAA levels were used, respectively. However, high MAA levels resulted in color changes. Therefore, MAA at low levels had a photoprotective effect for natamycin stability in cow milk exposed to light in refrigerated glass containers.

Key words: natamycin, milk, mycosporine-like amino acid

Short Communication

According to Potts et al. (2017), milk consumption in the United States has declined in recent years, possibly because consumers experience off flavors in fresh milk as a result of light-induced oxidation reactions. In general, fluid milk is commercialized in packages that allow light to penetrate and react with it. Therefore, milk quality and flavors degrade in as fast as 4 h of

light-emitting diode (**LED**) light exposure under commercial conditions (Potts et al., 2017).

Johnson et al. (2015) reported that light-induced oxidation reactions in milk negatively affect odor and flavor, which ultimately reduces shelf-life. Photooxidation can take place in the presence of light in UV and visible wavelength regions (Webster et al., 2009) and rapidly affects consumers' milk acceptability (Duncan and Webster, 2010; Potts et al., 2017) and aroma profile (van Aardt et al., 2005).

Food packaging is a practical approach to protect food from light damage. However, consumers prefer to see the produce to evaluate quality; therefore, many foods are packaged in clear containers (Doyle, 2004), but clear or translucent packaging allows exposure to light, increasing the risk of light-induced degradation or oxidation (Duncan and Webster, 2009). Therefore, food additives are incorporated into food for functional purposes, and in many cases these ingredients can also be found occurring naturally in some foods, such as natamycin (Lindsay, 2008; Mattia et al., 2016).

Natamycin is a fungicide of the polyene macrolide antibiotic group produced by *Streptomyces natalensis* and related species. It is generally recognized as safe (GRAS) and has been used as a natural food additive to control the growth of yeast and mold (Mattia et al., 2016). Natamycin is a food preservative approved in the European Union, and in the United States it is permitted to be used as a preservative in dairy products (20 mg/kg), such as cheeses, shredded cheese, cottage cheese, cream cheese, sour cream, and yogurt (Delves-Broughton, 2014). However, natamycin is very sensitive to UV light (Thoma and Kübler, 1998) and photodegradation leads to biological inactivation (Dekker and Ark, 1959). Therefore, in order for natamycin to express its preservation properties in dairy products stored in transparent packaging, something must be done to control natamycin photodegradation.

Mycosporine-like amino acids (**MAA**) participate in the biochemical defenses against exposure to high

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fluxes of UV light in a wide variety of marine organisms (Fleischmann, 1989; Shick et al., 1992). Mycosporine-like amino acids are polar compounds with high molar absorptivity ($\epsilon = 28,000\text{--}50,000\text{ M}^{-1}\cdot\text{cm}^{-1}$; Nakamura et al., 1982; Cardozo et al., 2007), which is strongly related to the MAA photoprotective effect, which is their primary function (Dunlap et al., 1998; Torres et al., 2006).

Recent work (Teixeira et al., 2018) has reported that solid-phase extraction (SPE) purified MAA have a significant photoprotective effect on natamycin in acidified beverages stored under commercial refrigerated conditions and exposed to either fluorescent and low LED light. However, the low pH (3.5) of this beverage might prompt natamycin degradation itself (G. H. A. Teixeira, unpublished data). These same authors reported that at natural green tea pH (5.4), natamycin was less subject to photodegradation (G. H. A. Teixeira, unpublished data). Therefore, the hypothesis of our current study is that MAA might have a photoprotective effect in cow milk due to its higher pH (6.4–6.6). Thus, the objective of this study was to evaluate the photoprotective effect of MAA in whole dairy cow milk stored under 2 lighting conditions: fluorescent and high output LED light.

The MAA extraction was carried out according to Yuan et al., (2009). Briefly, 40 g of freeze-dried certified organic red dulse (*Palmaria palmata* L.) from Maine Coast Sea Vegetables Inc. (Hancock, ME) was extracted with 480 mL of methanol overnight, with 3 successive overnight extractions. Methanol extracts were filtered using Whatman no. 5 paper filters and combined. Methanol was removed by rotary evaporation (Rotavapor R-3000, Buchi Corporation, New Castle, DE) at 40°C and 50 rpm, until about 100 mL remained. The remaining methanol was washed twice with 100 mL of n-hexane, and hexane solutions were discarded. The methanol phase was extracted with a mixture of water (100 mL) and ethyl acetate (100 mL). The lower methanol-H₂O layer was retained and extracted with 150 mL of 1-butanol. The upper methanol-H₂O-1-butanol phase was concentrated by rotary evaporation (50–55°C) to obtain a light brown powder. This extraction procedure was repeated 3 times.

The MAA extracts were characterized based on UV-visible absorbance spectra obtained using a Shimadzu spectrophotometer (model UV-2500, Shimadzu Corporation, Tokyo, Japan). High-performance liquid chromatography with diode-array detection (DAD) analysis was also carried out according to Yuan et al. (2009) using an Agilent 1100 Series high performance liquid chromatogram (Agilent Technologies Inc., Palo Alto, CA). The injection volume was set to 2 μL and

a Macherey-Nagel Nucleosil (Oensingen, Switzerland) 100–5C18 (250 $\times$ 4.6 mm, 5 μm particle size, 100 Å pores) column was used at 30°C. Ultra-performance liquid chromatography with mass spectrometry (UPLC-MS) analysis was also performed (Yuan et al., 2005). A Waters Acquity UPLC H-Class system with QDA detector and Empower software (Milford, MA) with an AccQ-Tag Ultra C18 (100 $\times$ 2.1 mm, 1.7 μm particle size, 130 Å pore size) column was used, and the analyses were performed in scan and single ion recording modes with the goal of identifying the MAA compounds tentatively identified with C-18 HPLC-DAD.

Whole HTST pasteurized cow milk samples were obtained in a local supermarket and natamycin was added at a concentration of 21 mg/L as used by Teixeira et al. (2018). Afterward, 15 mL of milk was filled into 20-mL glass bottles, capped tightly, and used immediately.

For the first experiment, clear glass flasks containing milk samples were stored in commercial display cases under fluorescent (model ONRB4, Hillphoenix, Conyers, GA) or LED (model ON5DMA8, Hillphoenix) light for up to 8 d at 4.0°C. The control no light group was covered with aluminum foil. Light intensity was measured using an Upertek MK350S (Miaoli County, Taiwan), and $354.9 \pm 70.9\text{ lx}$ was recorded with fluorescent and $2,131.7 \pm 354.9\text{ lx}$ with LED light ($n = 20$). The positions of the glass flasks were randomly rotated every 24 h and flasks containing milk were taken from the storage on d 0, 2, 4, and 8 for natamycin analysis using the procedure described by Paseiro-Cerrato et al. (2013). An external standard calibration curve was used to quantify the natamycin which was reported as milligrams per liter. The natamycin retention was expressed as a percentage of the initial concentration (d 0). The experiment was designed as a completely randomized design with 3 light treatments (control-foil, LED, and fluorescent light) and 4 repeated measurements (0, 2, 4, and 8 d) with 2 replications. The data were subjected to ANOVA and the treatment means were compared using Tukey's test at a significance level of $P < 0.05$. The data were analyzed using the software Statistica (Release 4.5, StatSoft Inc., Tulsa, OK).

For the second experiment, MAA were added into milk samples to determine if MAA prevents natamycin photooxidation. Three different levels of MAA were used based on UV-visible absorbance at 330 nm: (1) control (0 – MAA), (2) low MAA (~ 4 UV-visible absorbance), and (3) high MAA (5 times the concentration to obtain ~ 4 UV-visible absorbance).

The glass flasks containing the milk samples were stored in a commercial display cabinet (model ON5DMA8, Hillphoenix) with LED light exposure. In pre-

vious work, under these conditions, rapid natamycin photodegradation occurs by 8 d at 4.0°C. The control group was stored in aluminum foil-covered glass flasks to avoid light. Light exposure intensity was measured 30 times using an Upertek MK350S (Miaoli County, Taiwan) and $1,280.7 \pm 658.9$ lx was recorded. The positions of the glass flasks were randomly rotated every 24 h and flasks containing milk were taken from the storage on d 0, 2, 4, and 8 for natamycin analysis according to Paseiro-Cerrato et al. (2013). The experiment was designed as a completely randomized design with 3 MAA concentrations (control, 0 MAA; low MAA, ~4.0 abs; and high MAA, ~20 abs) and 4 repeated measurements (0, 2, 4, and 8 d) with 2 replicates. The data were subjected to ANOVA, and if main effects were significant, the means were separated using Tukey's test at a significance level of $P < 0.05$. The data were analyzed using the software Statistica (Release 4.5, StatSoft Inc.).

Although 21 mg/L of natamycin was added into milk samples, we could only determine 8.9 ± 3.0 mg/L by HPLC in milk samples, exposed or not to light, at d 0. Based on this concentration, the percentages were calculated for each evaluation period. Therefore, natamycin solubility in milk appears much lower than in distilled water (20–50 mg/L) or matrix differences between water and milk affected extraction.

A significant interaction ($P < 0.05$) between light sources on natamycin retention was observed (Figure 1A). When milk samples were protected from light by covering the glass flasks with aluminum foil, no significant natamycin degradation was observed (Figure 1A). On the other hand, when milk was exposed to fluorescent or LED light, natamycin was rapidly degraded and the final concentration was of only $43.4 \pm 1.2\%$ and $17.1 \pm 0.9\%$ of the original level, respectively (Figure 1A).

Although natamycin shows good stability in foods in a pH range of 5 to 9 (Raab, 1972), natamycin is very sensitive to UV light (Thoma and Kübler, 1998) and photolytic reactions leads to its biological inactivation (Dekker and Ark, 1959). Even though milk has a pH around 6.4 to 6.7, this range did not result in natamycin stability when milk was exposed to light.

According to Potts et al. (2017), retailers are replacing fluorescent lights with LED lights to reduce energy costs. In one study, LED light (4,000 lx) also caused fewer nutritional modifications due to light-induced oxidation after 24 h of light exposure compared to fluorescent light (2,200 lx; Brothersen et al., 2016). However, LED light (2,132 lx) caused significantly more natamycin degradation than fluorescent light (355 lx; Figure 1A). Because the differences in the lighting includes lux intensity as well as spectral wavelengths,

we cannot determine which of these caused the degradation difference in our work. However, because we used commercial display cabinets, the reported lighting would be expected to be similar in real world applications.

To protect natamycin from photooxidation, MAA were incorporated into milk samples stored under LED light because exposure under these conditions results in fast natamycin degradation (Figure 1A). Similarly, in the absence of light (light-blocked control), less natamycin photooxidation was observed, but a significant reduction in natamycin concentration on d 4 was observed in milk samples with no added MAA, which was somewhat different from the first experiment in which no significant natamycin degradation took place (Figure 1B). By the end of the storage period, natamycin retention was of 76.6 ± 2.8 , 93.9 ± 1.1 , and $108.7 \pm 1.4\%$ of the original natamycin level when milk samples were protected from light (control) and at low (~4 abs) and high (~20 abs) MAA levels, respectively (Figure 1B). Teixeira et al. (2018) reported that the incorporation of low (~2 abs) and high (~4 abs) MAA levels resulted in significant natamycin photoprotection in acidified beverages (green tea infusion) when MAA were fractionated using SPE. However, the incorporation of MAA without SPE purification in milk samples presented a natamycin photoprotective effect, different from acidified green tea beverages.

When milk samples were exposed to LED light, natamycin content decreased sharply with just $9.7 \pm 1.0\%$ of the original level remaining after 8 d at 4.0°C (Figure 1B). On the other hand, when MAA were incorporated, 82.2 ± 4.3 and $92.2 \pm 0.2\%$ natamycin retention was observed at low (~4 abs) and high (~20 abs) MAA levels, respectively (Figure 1B). The MAA incorporation into acidified beverages resulted in faster natamycin photooxidation (G. H. A. Teixeira, unpublished data), but did not happen in milk. Therefore, MAA interacting with milk components may lead to natamycin photoprotection and it should be interesting to investigate if MAA also have an effect on light-induced oxidation in milk samples exposed to light.

The chemical composition of the MAA extract was similar to Yuan et al. (2009). The UV-visible spectra showed only 1 peak at maximum absorption ($\lambda_{\max}$) at 330 nm, which corresponds to the presence of MAA (Carignan et al., 2009). The HPLC-DAD chromatogram presented 6 peaks (Figure 2A). The MAA identification was carried out by UPLC-MS total ion chromatogram at 245.00, 333.10, 289.10, 303.10, 347.10, and 285.10, which corresponded to known MAA.

Incorporation of MAA into milk has a photoprotective effect on natamycin during storage under fluorescent and LED light and could conceivably be used as

Figure 1. Natamycin retention (%) in cow milk exposed to different light sources [control—foil, fluorescent, and light-emitting diode (LED) light] for 8 d at 4.0°C (A, experiment 1), and with mycosporine-like amino acid (MAA) incorporation at different levels (control—without MAA, low MAA ~4.0 abs, and high MAA ~20 abs) and exposed to LED light for 8 d at 4.0°C (B, experiment 2). The bars represent the SD of 2 replicates. abs = absorbance. Average values with the same uppercase letters (A–C) within storage period are not statistically different by Tukey's test ($P < 0.05$). Average values with the same lowercase letters (a–c) within treatment are not statistically different by Tukey's test ($P < 0.05$).

an additive to protect milk from light-induced oxidation reactions. However, the interactions between MAA and milk components are still an open field of study, and future studies should address mechanisms.

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Figure 2. (A) High-performance liquid chromatography-diode-array detection chromatogram of natamycin and (B) mycosporine-like amino acids (MAA) in cow milk samples.

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