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Evaluation of *in vitro* bioassays as a screening tool to monitor chemical hazards in cow's milk

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ABSTRACT

Studies on cow's milk have mainly focused on analyzing specific chemical groups and natural components. Therefore, in this study, we evaluated if effect-based *in vitro* methods could be used as a screening tool to monitor chemical hazards in milk. In total, 32 milk samples were collected from a Swedish dairy company throughout one year. These samples included conventional and organic semi-skimmed as well as raw milk. The milk samples were tested in five *in vitro* methods covering eight endpoints. These endpoints included cytotoxicity, endocrine disruption (estrogen/androgen induction/inhibition), aryl hydrocarbon receptor activity, oxidative stress and DNA damage. Estrogen and androgen receptor inhibition, in addition to aryl hydrocarbon receptor activity, were the most responsive endpoints, where 10 to 13 out of the 32 milk samples were bioactive. Organic and conventional milk showed no major differences. Overall, no or only low activities were observed in milk samples in the remaining *in vitro* assays, which is a promising result with regard to applying effect-based methods as a screening tool. Concerning the most responsive assays, more research is needed to understand the normal background variations before they can be used as a screening tool for chemical hazards in milk.

1. Introduction

Chemical pollutants have been extensively studied in matrices like surface, drinking and wastewater (Escher et al., 2013; König et al., 2017; Lundqvist et al., 2021; Oskarsson et al., 2021). Multiple studies have shown that the most often analyzed and/or well-known pollutants only explain a small fraction of the toxicity observed within the *in vitro* methods (Escher et al., 2013; König et al., 2017; Oskarsson et al., 2021). Thus, relying solely on chemical analysis of a limited number of individual substances provides an inadequate picture of the hazards posed by chemical pollutants. Since milk and milk products are food groups that are consumed by numerous people, it is important to have a good control system in place to ensure that these products are not contaminated. The main causes of contamination are via feed and water (Schulz et al., 2005).

Most research efforts in milk monitoring have focused on quantifying specific chemical groups, natural compounds and the composition of the milk (Foroutan et al., 2019; Di Bella et al., 2020; Hasan et al., 2022; Róin et al., 2023), but there is scarce information on the overall biological effects of the total milk chemical exposome that potentially can be related to adverse health effects, and how these effects may vary throughout the year. This underlines the necessity to adopt a holistic approach, where the effects of known, unknown and mixtures of biologically active chemicals are efficiently evaluated. *In vitro methods*, also referred to as effect-based methods, yield information about the modes of action of chemicals and indicate if there are chemicals of concern in a sample. These methods can be used early in the hazard assessment (Escher et al., 2021b). Consequently, we wanted to apply a similar approach with the aim to evaluate if *in vitro* bioassay methods could be used as a screening tool to monitor chemical hazards in cow's milk.

The present study, therefore, used a panel of five *in vitro* methods, all closely linked to toxicity pathways of high relevance to human health. We wanted to investigate the background levels of bioactive compounds in Swedish milk samples and see if any differences between organic and conventional raw and semi-skimmed milk could be quantified. Additionally, we also wanted to explore if there were any seasonal variations in cow's milk. We hypothesized that higher activities in the cow's milk may be observed in the mandatory grazing period of the cow (i.e. outdoor period), as the consumption of grass and unintentional ingestion of soil increases, which can contain chemicals like polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (McLachlan, 1993; Hasan et al., 2022). These chemical groups are known to

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increase the activity of AhR. Furthermore, we also hypothesized that the estrogen receptor activities potentially could be altered during the consumption of clover when grazing, due to the phytoestrogen content (Róin et al., 2023), that is if the phytoestrogens are broken down during the conservation of the silage. The endpoints focused on were cytotoxicity, endocrine disruption (estrogen/androgen receptor induction/inhibition), xenobiotic metabolism (aryl hydrocarbon receptor activity), oxidative stress (in the form of Nrf2 activity) and DNA damage (micronucleus test).

2. Materials and methods

2.1. Chemicals and solvents

The solvents acetonitrile (75-05-8, ≥99.9%) and formic acid (64-18-6, ≥98%) were purchased from Sigma-Aldrich. Methanol (67-56-1, ≥99.8%) was supplied from VWR. Ultrapure water (Milli-Q®) was sourced from a Millipore® facility system using a 0.22 μm filter.

Dimethyl sulfoxide (DMSO, CAS 67-68-5 >99.9%), 5α-androstan-17β-ol-3-one (DHT, CAS 521-18-6, ≥97.5%), β-estradiol (CAS 50-28-2, ≥98%), hydroxyflutamide (OHF, CAS 52806-53-8, ≥98%), methoxychlor (CAS 72-43-5, 98.7%), raloxifene hydrochloride (Ral, CAS 82640-04-8), tamoxifen (CAS 10540-29-1, ≥99%), 2,3,7,8-tetrachlorodibenzo-p-dioxin solution (TCDD, CAS 1746-01-6), tert-butylhydroquinone (tBHQ, CAS 1948-33-0, 97%) and mitomycin C (MMC, CAS 50-07-7) were acquired from Sigma-Aldrich.

2.2. Sample preparation and extraction

Representative milk samples were collected monthly from the largest dairy company in Sweden between June 2020 to May 2021 (weeks 26, 2020 to 21, 2021) and consisted of in total 32 samples; organic semi-skimmed milk (n = 4), conventional semi-skimmed milk (n = 12), organic raw milk (n = 4) and conventional raw milk (n = 12). Organic milk was collected every third week, starting from week 26 2020 and ending at week 12 2021. The raw milk was pooled from multiple farms around the dairy plant and was collected before any processing occurred. Normally, raw milk has a fat content of 4.3%. The raw milk arrived in sterile polyethylene terephthalate (PET) sampling bottles with blue polypropylene (PP) caps (VWR®, #3310269). Semi-skimmed milk, on the other hand, had a fat content of 1.5% and was homogenized as well as pasteurized. These were delivered in commercially available coated paperboard cartons. Directly after packaging, milk samples were frozen (−20 °C) until the sample preparation started.

A similar method for milk sample preparation and extraction was applied as the one developed by Waters (Huang et al., 2015). Each milk sample was mixed by inversion a few times prior to opening. For 50 mL of milk, 200 mL of 0.2% formic acid in acetonitrile was added and mixed to precipitate proteins. The samples were then centrifuged for 30 min at 5000 rpm and supernatants were collected for solid-phase extraction (SPE). The 3 cc Oasis PRiME HLB Cartridge (Oasis, #186008056) was conditioned with 0.2% formic acid in acetonitrile. Thereafter, the supernatants were loaded onto the cartridge and collected. The cartridge allowed matrix interferences like phospholipids and fats to efficiently be removed from the milk. The fast and effective modified SPE method allows acidic, basic and neutral compounds to be retrieved with high recoveries (Huang et al., 2015). After collection, the samples were filtered using a 0.22 μm filter. Evaporation to dryness occurred on the TurboVap II Evaporation System (Biotage) and samples were dissolved in 0.5 mL of 5% methanol in Milli-Q® water (5% MeOH/H_20).

The concentrations of the milk samples were expressed as the relative enrichment factor (REF). Milk samples were 100× enriched during the extraction procedure and 100× diluted in the *in vitro* assays, resulting in the highest concentration of 1. The final plate concentrations ranged from REF 1.00 to 0.02. REF <1 represents diluted samples. The final concentration of the milk samples depended on the cell viability results.

Three solvents blanks treated in the same way as the samples, but without any milk, were also prepared and tested. All samples were stored at −20 °C until analysis.

2.3. In vitro test methods

A panel of *in vitro* methods, all closely linked to toxicity pathways of high relevance to human health, was applied to the milk samples. These covered specific action modes (estrogen/androgen receptor induction/inhibition, aryl hydrocarbon receptor induction), non-specific (cytotoxicity) and reactive toxicity (micronucleus formation; MN, oxidative stress). Additional details of the *in vitro* methods and cell maintenance are found in the Supplementary Information (SI 1–3).

Each run was validated by using an assay-specific reference compound to generate a dose-response curve. Further information on the standards as well as yielded effect concentration (EC) and inhibitory concentrations (IC) can be found in the Supplementary Information (Table S1).

The vehicle controls consisted of 5% methanol in Milli-Q® water for the milk samples and DMSO for each standard. The standards tested were tBHQ, TCDD, DHT and β-estradiol, for oxidative stress, AhR response, induction of androgen as well as estrogen receptors, respectively. Hydroxyflutamide was used for the inhibitory response of the androgen receptor, while raloxifene was used for the inhibitory estrogen receptor activity. The positive controls included tamoxifen (ER inhibitory response), methoxychlor (ER induction response) and mitomycin C (genotoxic response).

2.4. Data analysis

Cell viability data were expressed as fold change compared to the vehicle controls, which was set as 100% for all *in vitro* tests apart from the micronucleus test. A reduction of more than 25% was defined as cytotoxic. For the micronucleus test, cytotoxicity was evaluated by staining the cells with ethidium monoazide stain (EMA), and a 4-fold increase of %EMA-positive events to the vehicle control was considered cytotoxic (Bryce et al., 2013; Laboratories, 2018).

The limit of detection (LOD) of all the *in vitro* endpoints was calculated to identify the concentration of the reference compound that induces three times the standard deviation (SD) of the normalized vehicle control, except for oxidative stress (Escher et al., 2021c). For oxidative stress, LOD was instead calculated as one plus three times the SD of the normalized vehicle control (Escher, Neale and Leusch, 2021c). Based on the LOD the cut-off was set to express the sample as bioactive.

The cut-off for oxidative stress, induction mode of the hormonal receptors and AhR response was set as the even number above the LOD. For the inhibitory mode of the hormonal receptors, the cut-off was set as the even number below the LOD (Table S1).

The data generated from the AhR and induction of hormonal receptors were normalized to the vehicle control, followed by being normalized to the maximum (max) effect of the corresponding standard. The inhibitory mode of the receptors was, on the other hand, first normalized to the unspiked vehicle control and then normalized to the max effect of the spiked vehicle control.

The bioanalytical equivalent concentrations (BEQ) were only calculated for bioactive samples to relate the effect of a known standard to the effect of a milk sample. It was derived by dividing the ratio of the effect concentration (EC) of the reference compound by the EC value of the sample, per the following equation (Escher et al., 2021a):

$$BEQ_{bioassay} = \frac{EC_{IR1.7}, EC_x \text{ or } IC_{30}\ (reference\ compound)}{EC_{IR1.7}, EC_x \text{ or } IC_{30}(sample)}$$

$$x = 10, 20$$

The standard error (SE) for BEQ for oxidative stress was calculated

according to the formulas (Escher, Neale and Leusch, 2021a):

$$EC_{IR1.7} = \frac{0.7}{slope}$$

$$SE(EC_{IR1.7}) = \frac{0.7}{slope^2} \times SE(slope)$$

For the remaining assays, where the effect was linear up to 30% of the max effect, the SE was derived by the equations (Escher et al., 2021a):

$$y = slope \times concentration$$

$$EC_x = \frac{y}{slope}$$

$$SE(EC_x) = \frac{y}{slope^2} \times SE(slope)$$

$$SE\left(BEQ_{bioassay}\right) = \sqrt{\frac{1}{EC_x(sample)^2} \times SE(EC_x(reference\ compound))^2 + \frac{EC_x(reference\ compound)^2}{EC_x(sample)^4} \times SE(EC_x(sample))^2}$$

Linear regression analysis was applied in GraphPad Prism version 9.5.0 (San Diego, California, USA), after normalizing the data to the vehicle control (fold change) to fit the oxidative stress data, as no max response exists (Escher et al., 2014). Nonlinear regression (log-logistic) analysis was used for the remaining standards with a four-parameter sigmoidal curve fit in GraphPad Prism (Table S1).

Data generated from the micronucleus test was first assessed in FCS Express 7 Flow Research Edition, then in GraphPad Prism version 9.5.0, where bioactivity was statistically evaluated by one-way ANOVA with Dunnett's Multiple Comparison test. Bioactive samples were defined by being statistically significant to the normalized vehicle control (p-value below 0.05).

3. Results and discussion

3.1. Bioactivities

3.1.1. Cytotoxicity testing

Generally, no cytotoxicity was observed in MCF7 AREc32, VM7Luc4E2 and TK6 cells in the concentration range tested (Figs. S1, S3 and S5). The only exception was raw milk at week 2 in VM7Luc4E2 cells, which exerted a high cytotoxic effect at REF 1 (Fig. S3). On the contrary, 22 out of the 32 milk samples were cytotoxic at the highest REF of 1 in the DR-EcoScreen cell line (Fig. S4), while 26 out of 32 milk samples were cytotoxic in the AR-EcoScreen GR-KO M1 cell line at REF 1 (Fig. S2). Two of these samples (W35 - raw milk, W2 – semi-skimmed milk) were cytotoxic down to REF 0.25.

The solvent blanks did not affect the viability (*data not shown*). Based

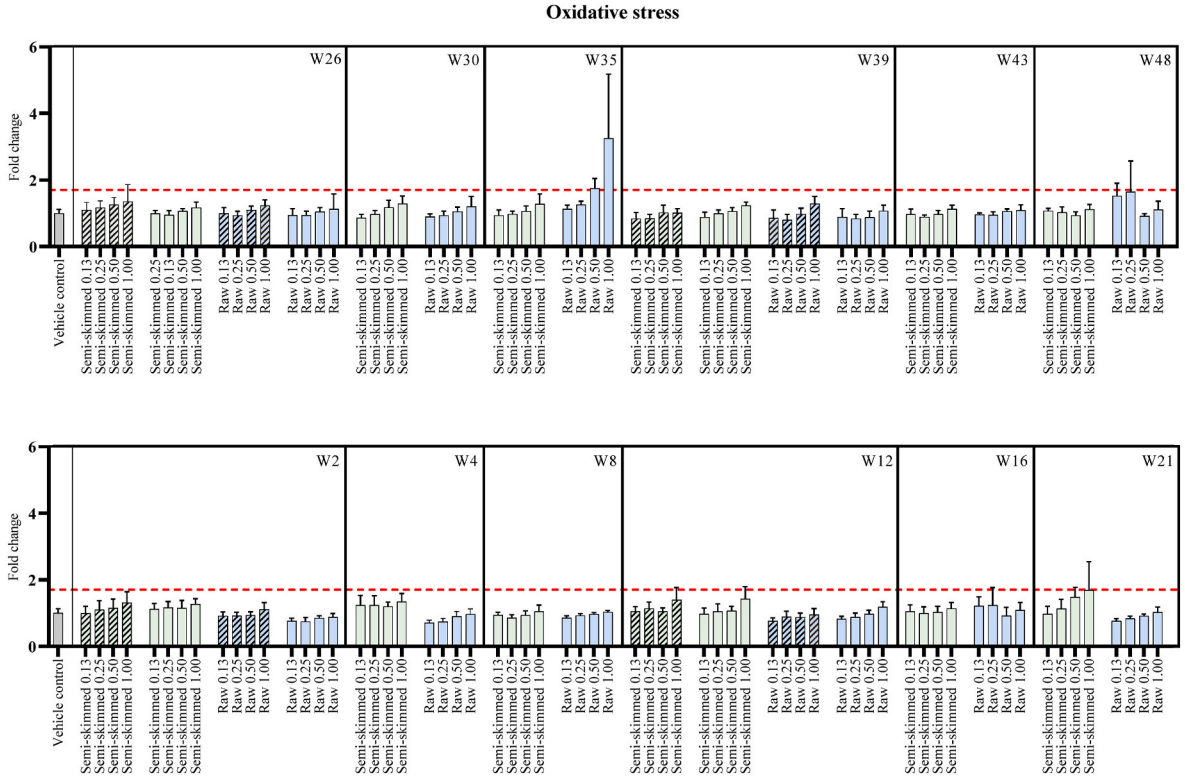

Fig. 1. Bioactivity of milk samples after exposure for 24 h in the oxidative stress *in vitro* assay. Semi-skimmed milk is defined by the green color, while raw milk is defined by the blue color. Organic milk is highlighted with stripe patterns (angled solid black lines). Concentrations are expressed as REF. Data are shown as mean ± SD from two independent experiments (n = 4 for milk samples/run, n = 8 for vehicle control/run). The red dotted lines mark the cut-off level of 1.7-fold change. "W" denotes the week the milk was taken. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

on these findings, non-cytotoxic concentrations were used for the remaining *in vitro* test methods.

3.1.2. Oxidative stress

Only one sample, raw milk at week 35, was bioactive in the oxidative stress assay (Fig. 1). Raw milk, at week 35, was bioactive at the two highest concentrations and reached a 3.3-fold change increase compared to the vehicle control (Fig. 1). The BEQ for the raw milk sample at week 35 was 4.92×10^{-6} M tBHQ equivalents (eq) (Table S2).

There are many differences between raw and semi-skimmed milk at week 35, besides the difference in fat content, the raw milk itself is not processed (i.e. pasteurized or homogenized). Also, as milk is pooled, the raw and semi-skimmed milk may not come from the same batch of milk. Additionally, they were also stored in different containers. Before the extraction, the semi-skimmed milk was stored in the commercially available coated paperboard carton while raw milk was stored in PET bottles. However, the reason why only one specific sample in our study deviated from the remaining PET-stored samples and/or semi-skimmed milk samples is not known. It may be possible that it was exposed to more light before being delivered, resulting in the degradation of light-sensitive protective molecules with antioxidant properties like vitamin A. Another hypothesis is that the fat-soluble substances are driving the oxidative stress and these exist at higher concentrations in raw milk.

One previous study has emphasized the importance when selecting the storage bottle, where for example higher lipid oxidation of homogenized whole milk (3.5% fat) was seen in PET bottles with increasing time (0–7 days of storage) compared to pigmented high-density polyethylene (HDPE) and coated paperboard cartons (Zygoura et al., 2004). Furthermore, the degradation of vitamin A was most pronounced for the clear PET bottles (51% loss), followed by pigmented PET (30% loss) and control samples consisting of coated paperboard carton (14%). These results illustrate that there can be changes in the composition of the milk samples depending on the type of storage bottle that is chosen (Zygoura et al., 2004). Regardless, we have earlier stored Milli-Q® water and tap water in the same type of PET bottles, as used in this study, for longer times and they did not show any activity of the assays tested (Lundqvist et al., 2021).

Mojica and Bisso (2021) reported increased total antioxidant capacity in commercially available non-fat chocolate milk using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay, while all the other commercially available milk samples (whole, non-fat and reduced fat) showed a similar antioxidant response.

The high antioxidant response in non-fat chocolate milk was hypothesized to be due to the increased content of polyphenols in cocoa powder (Mojica and Bisso, 2021). Nonetheless, it is important to highlight that the study mentioned above did not extract the milk samples, which we did in our study to remove the milk matrix that could interfere with the assay.

Generally, we did not observe oxidative stress in the samples, which is of great benefit if the method is to be used as a screening tool. Oxidative stress was observed in one case, which also is good from a methodological standpoint because it demonstrates that we can capture oxidative stress-causing substances, if present in the milk.

3.1.3. Genotoxicity

As oxidative stress is one of the multiple mechanisms that can cause genotoxic effects, raw milk at week 35 was hypothesized to potentially be genotoxic. Even though only one sample was recognized to be bioactive in the oxidative stress assay, four conventional raw milk samples, close to the cut-off limit of the oxidative stress assay, were tested at two concentrations (REF 1.00 and 0.50) in the micronucleus test to evaluate their genotoxic potential. However, none of the tested milk samples proved to be genotoxic (Table 1).

Anthropogenic pollutants like PCB congeners, polybrominated diphenyl ethers (PBDEs) and/or PAHs have been detected in raw as well as commercially available whole and fat-free milk samples (Chen et al., 2017; Di Bella et al., 2020; Hasan et al., 2022). The presence of these pollutants within milk was hypothesized to be related to the feedstuff as they may consume contaminated feed through grass, maize and soil. Both PCBs and PAHs are classified as group 1 carcinogens by the International Agency for Research on Cancer (IARC), where several of the chemicals need metabolic activation to cause their DNA-damaging effects (IARC, 2010, 2016). Since no metabolic components were added in this study, future studies need to investigate the potential genotoxic effect of metabolically active chemicals in milk. Still, the non-existing background activity of genotoxicity observed in milk samples is promising in regard to the idea of using effect-based methods as screening tools.

Table 1
Summary of micronucleus test results from two independent runs (n = 4 for samples/run, n = 3–4 for vehicle controls/run). Concentrations of the milk samples and vehicle controls are expressed as REF.

Milk sample	Week (year)	REF	MN formation	
			Average %MN ± SD	Average MN fold change ± SD
Raw milk	35 (2020)	1.00	0.16 ± 0.04	0.68 ± 0.16
		0.50	0.14 ± 0.04	0.62 ± 0.16
	48 (2020)	1.00	0.22 ± 0.07	0.94 ± 0.29
		0.50	0.24 ± 0.04	1.05 ± 0.17
	12 (2021)	1.00	0.16 ± 0.05	0.68 ± 0.21
		0.50	0.14 ± 0.03	0.60 ± 0.14
	21 (2021)	1.00	0.11 ± 0.04	0.47 ± 0.17
		0.50	0.15 ± 0.02	0.64 ± 0.09
Vehicle control				
5% MeOH/ H_20	N/A	1.00	0.23 ± 0.07	1.00 ± 0.28
Milli-Q®		1.00	0.21 ± 0.05	1.00 ± 0.26
Positive control				
MMC	N/A	200 nM	1.08 ± 0.37[a]	5.17 ± 1.75[a]
		100 nM	0.65 ± 0.18[a]	3.10 ± 0.88[a]

[a] Samples that were statistically significant from its vehicle control are marked with an asterisk (p-value <0.0001).

3.1.4. AhR

In total, 12 out of 32 milk samples were bioactive in the AhR assay, of which four samples were semi-skimmed milk and the remaining eight were raw milk (Fig. 2). All bioactive samples demonstrated activity at their highest REF in a dose-related manner, except for raw milk at week 4 that was bioactive from REF 0.50. The highest REF of this particular sample was below the cut-off limit and it is likely related to undetected cytotoxicity and it was thus omitted from the BEQ calculation.

Raw milk at week 16 obtained the highest efficacy, reaching 19% of the max effect and was bioactive down to REF 0.13 (Fig. 2). Organic raw milk was slightly more bioactive than organic semi-skimmed milk, as seen at weeks 26 and 12, which potentially could be explained by the fact that raw milk has higher fat content than semi-skimmed milk, and dioxin as well as dioxin-like compounds are known to associate with fat. The organic semi-skimmed milk was only found to be active at week 2 (Fig. 2). The BEQs ranged from 4.99×10^{-13}–2.60×10^{-12} M TCDD eq (Table S2). The BEQ values were recalculated into TCDD eq per gram fat in the milk, this corresponds to 3.74 to 19.47 ρg TCDD/g fat for raw milk and 14.80 to 24.27 ρg TCDD/g fat for semi-skimmed milk.

The European Union (EU) has defined a max level for the sum of dioxins, which is 2.0 ρg World Health Organisation (WHO)-polychlorinated dibenzo-p-dioxins/polychlorinated dibenzofurans (PCDD/PCDFs)-TEQ/g fat (European Commission, Directorate-General for Health and Food Safety, 2023). It should, however, be noted that the BEQ values measured in our AhR assay are not directly comparable to the WHO-TEQ, even though they both are expressed as TCDD

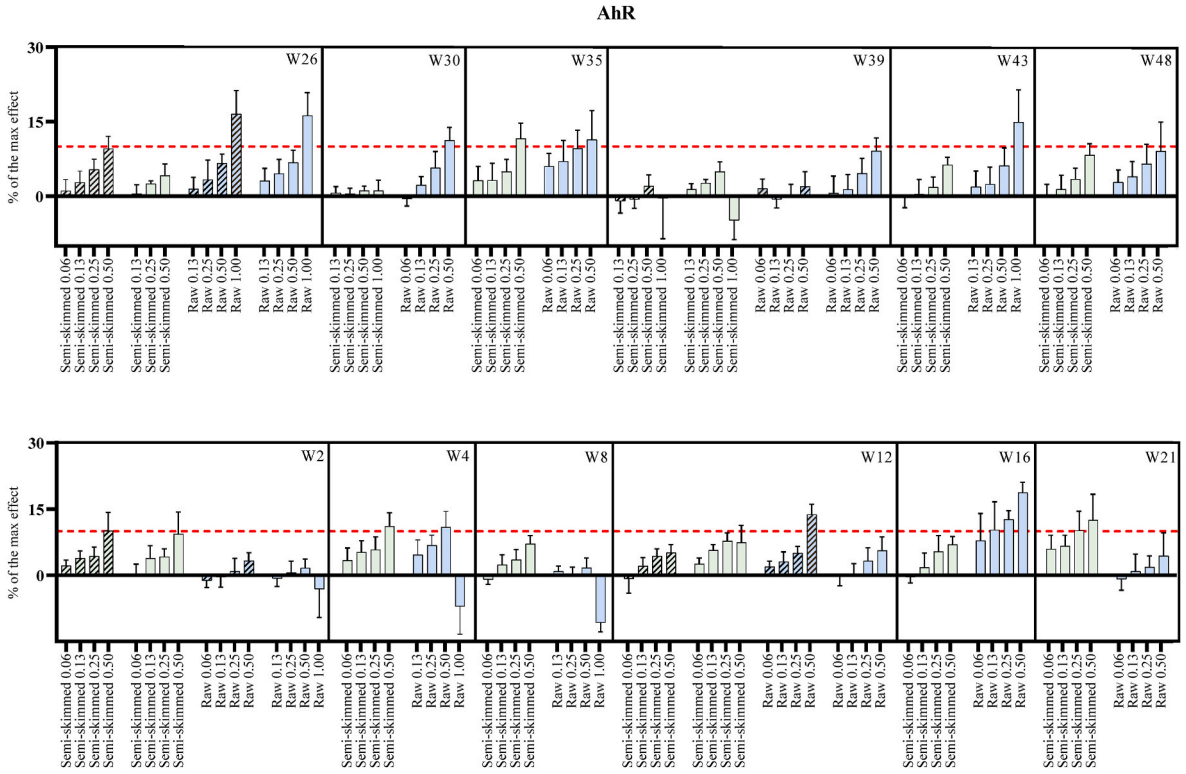

Fig. 2. Induction of the aryl hydrocarbon receptor (% of the max effect of the reference compound) after exposure for 24 h to milk samples. Semi-skimmed milk is defined by the green color, while raw milk is defined by the blue color. Organic milk is highlighted with stripe patterns (angled solid black lines). Concentrations of the milk samples are expressed as REF. Data represent mean ± SD from two independent experiments (n = 2–4 for samples/run, n = 8 for vehicle control/run). Samples with activity above the cut-off limit, represented by the red-dotted line, were defined as bioactive. "W" denotes the week the milk was taken. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

equivalents. The WHO-TEQ system is based on a principle to potency-scale a set of predefined individual chemicals for which the concentrations in a sample have been measured, and then calculate the potency-scaled sum concentrations, expressed as TCDD equivalents. The BEQ values measured in our AhR bioassay are the sum biological effects of both the dioxin and dioxin-like compounds covered by the WHO-TEQ system, and the thousands of other chemicals that have been reported to activate AhR. Hence, it could be expected that the BEQ value of a sample is higher than the WHO-TEQ value. To elucidate the fraction of the BEQ value that is constituted of the WHO-TEQ substances in a sample, parallel chemical analysis for the compounds covered by the WHO-TEQ system would be needed.

Both Mayilsamy et al. (2022) and Chou et al. (2008) evaluated PCDD and PCDFs in bovine milk, where the latter study showed levels below the earlier set threshold limit of 3 ρg WHO-TEQ/g fat (European Commission, Directorate-General for Health and Food Safety, 2006). The former study, on the other hand, retrieved total dioxin values of 0.03–7.33 ρg TEQ/g fat (Mayilsamy et al., 2022). Higher concentrations of dioxin-like compounds were thought to be attributed to the greatly populated areas and industrialized districts.

Contamination of dioxin in the soil, as well as grass, is well known (Schulz et al., 2005) and the replacement of feed at contaminated sites, especially hay, has reduced the contamination levels in milk (Bertocchi et al., 2015). Noteworthy, the sample preparations and extraction procedures used between the above-discussed studies and this study are dissimilar, which unquestionably will impact which chemicals that are captured and their effect(s).

We observed AhR activity in almost half of the samples, further studies are needed to understand whether the background variation of bioactive substances is because of natural compounds or anthropogenic contaminants.

3.1.5. Androgen receptor

All samples were below the cut-off limit in the androgen induction assay, meaning that none of the samples were bioactive (Fig. S6). Courant et al. (2007) reported overall lower concentrations of free androgens (dehydroepiandrosterone, α-testosterone and 4-androstenedione) in commercially available skimmed and half-skimmed milk compared to whole milk, where quantification of α-testosterone on average was 31.8 ng l^{-1} in skimmed milk and 51.3 ng l^{-1} in half-skimmed milk, while whole milk contained up to 78.1 ng l^{-1} on average. However, questions regarding analyses of phytoestrogens have been raised for extraction procedures using hydrolytic enzymes originating from *Helix pomatia* (Bláhová et al., 2016). It was seen that the use of this hydrolytic enzyme overestimated phytoestrogen content in milk, due to potential enzyme contamination, which also could be of importance for phytoandrogens.

Androgen receptor inhibition was observed in a dose-related manner for several of the milk samples (Fig. 3). Semi-skimmed milk at weeks 35, 12, 16 and 21, in addition to raw milk at weeks 39, 2, 4, 12, 16 and 21, were bioactive (Fig. 3). Semi-skimmed milk at week 16 obtained the highest BEQ value of 1.42×10^{-7} M OHF eq (Table S2). Studies on the androgen receptor-inhibitory activities in milk are very limited, highlighting that more research is needed to understand if the background levels are due to pollutants or naturally occurring chemicals.

The lack of response in the androgen induction assay is a promising finding, as it increases the chances of detecting contaminants that activate this parameter, compared to the situation with the inhibition of the androgen receptor where the milk itself was bioactive throughout the year and could mask the effects from the contaminants. Thus, more research is needed for the inhibitory mode of the androgen receptor.

3.1.6. Estrogen receptor

None of the milk samples induced the estrogen receptor after 24 h of

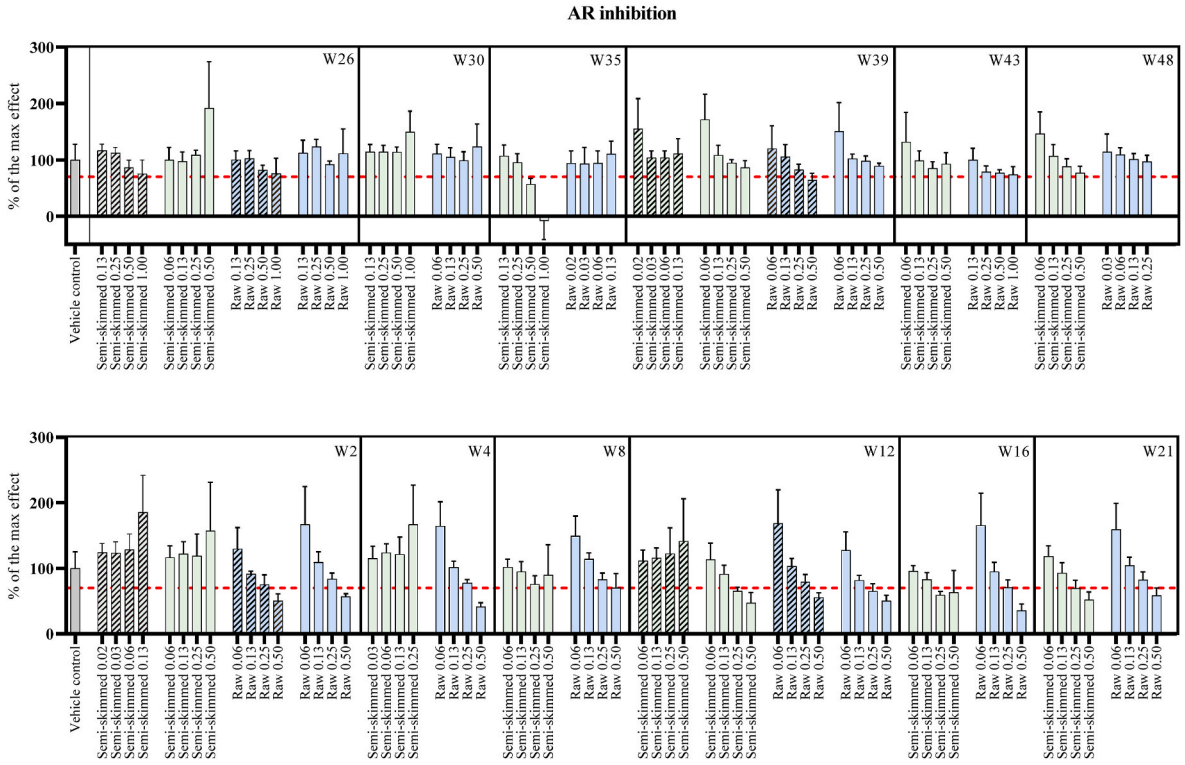

Fig. 3. Inhibition of the androgen receptor (% of the max effect of the reference compound) after exposure for 24 h to milk samples. Semi-skimmed milk is defined by the green color, while raw milk is defined by the blue color. Organic milk is highlighted with stripe patterns (angled solid black lines). Concentrations of the milk samples are expressed as REF. Data represent mean ± SD from two independent experiments (n = 2–4 for samples/run, n = 6–8 for vehicle control/run). Samples with activity below the cut-off limit, represented by the red-dotted line, were defined as bioactive. "W" denotes the week the milk was taken. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

exposure (Fig. S7). Dose-related inhibition of the estrogen receptor, on the other hand, was detected for a few semi-skimmed and raw milk samples between weeks 4–21 (Fig. 4). The inhibitory activity was most pronounced for semi-skimmed milk at week 21, reaching 44% effect at the highest REF, followed by 68% at REF 0.50 (Fig. 4). The organic milk lacked activity at all weeks, apart from week 12 where semi-skimmed milk was bioactive at REF 1. The BEQ ranged from 1.78×10^{-9}–3.20×10^{-9} M Ral eq (Table S2).

There is great potential to use effect-based methods as a screening tool, in regards to induction of the estrogen receptor, as low background activity was observed.

In agreement with the androgen inhibitory assay, the inhibitory effects of the estrogen receptor are not currently well reported in other studies. The studies existing mainly focus on the identification of specific phytoestrogens (Antignac et al., 2003; Steinshamn et al., 2008; Mustonen et al., 2009; Njåstad et al., 2014), the transfer of phytoestrogens into the milk (Mustonen et al., 2009) or seasonal variations of phytoestrogen in milk (Róin et al., 2023). However, it should be noted that, as previously stated, several studies may have overestimated the phytoestrogen concentration in the milk due to enzyme contamination (Bláhová et al., 2016). Regardless, the variation of phytoestrogenic compounds in milk between different areas in the world can be explained by the different plant species grazed by the cow, farm management systems (conventional, biodynamic and/or organic) and seasonal variations (Róin et al., 2023). Nevertheless, additional research needs to be conducted to understand the impact of the background levels of the inhibitory estrogen assay.

3.2. Monitoring using in vitro assays

This work utilized five *in vitro* methods covering eight toxicological endpoints to detect bioactivities in Swedish milk over one year. In general, no or low bioactivities were displayed for induction hormonal activities (ER/AR), genotoxicity and oxidative stress, while the activities of AhR and inhibition of the hormonal receptors were found to be more commonly occurring in the milk samples. None of the solvent blanks showed an effect in the eight endpoints tested (*data not shown*).

Interestingly, inhibitory estrogen activities were not observed until week 4 and the inhibition continued until week 21. Nearly all samples (10/32) between these weeks were bioactive only at the highest REF with relatively similar activities between the semi-skimmed and raw milk samples. It therefore appears to be seasonal differences in the presence of antiestrogens in the milk and it indicates that repeated sampling is of importance to understand the variations. It would be beneficial in the future to possibly perform effect-directed analysis to identify if the driving chemicals are of natural origin or pollutants (Brack et al., 2016), in a similar way that has been conducted by Hashmi et al. (2018) on wastewater. With this being said, AhR activity and inhibitory modes of action on the androgen receptor, appear to be even the whole year. These results confirm the value to conduct a follow-up of the present study and further develop *in vitro* methods to detect potentially hazardous chemicals within cow's milk. Such a follow-up study could further be enhanced by the inclusion of recovery experiments with the reference compound for each assay before and after the extraction. Additionally, the inclusion of an additional clean-up step with silica would be beneficial, in order to see how much of the AhR activity is driven by persistent chemicals.

The assays where activity was seen in a few samples would be the best candidates to follow-up because the background level of these substances is low. This means that we more easily could detect any contaminants since the signal would not be disturbed by a high background activity. The endpoints with higher occurring activities are more challenging to use as a screening tool, as we currently do not know if the background levels are due to pollutants (like dioxins), foreign

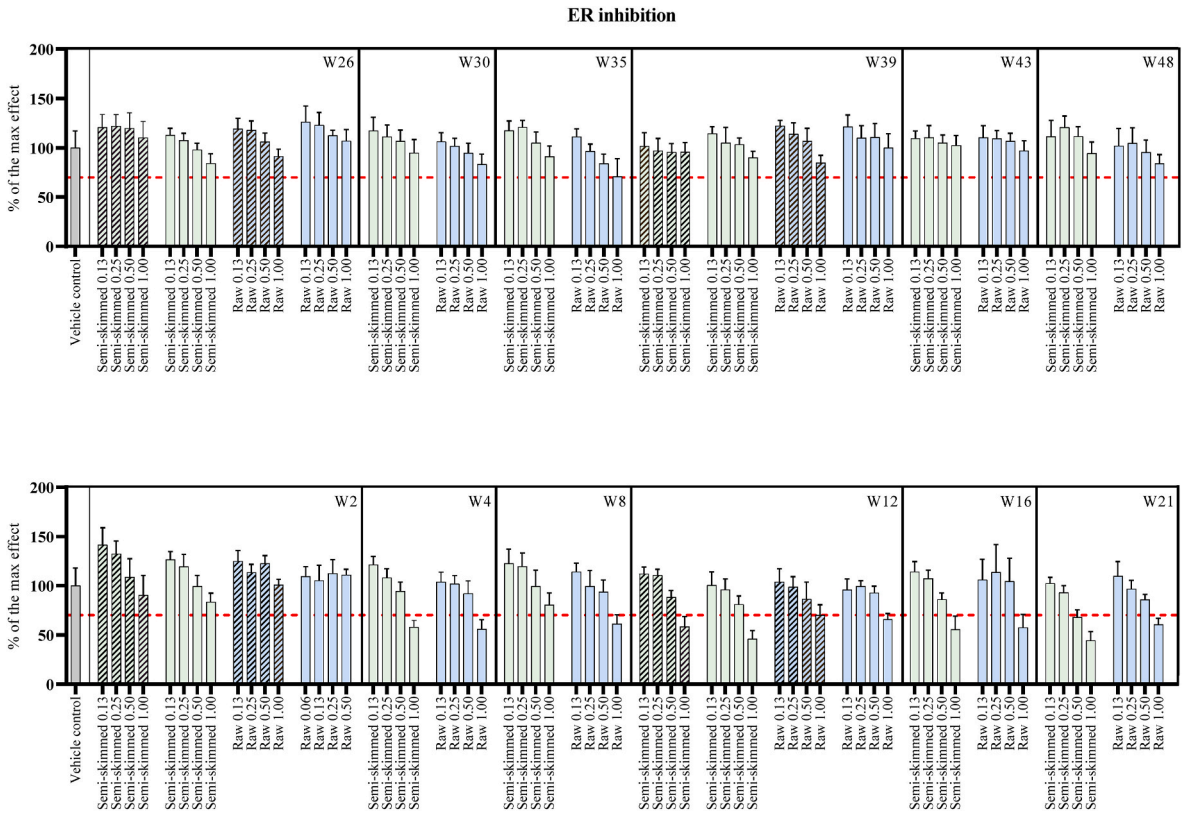

Fig. 4. Inhibition of the estrogen receptor (% of the max effect of the reference compound) after exposure for 24 h to milk samples. Semi-skimmed milk is defined by the green color, while raw milk is defined by the blue color. Organic milk is highlighted with stripe patterns (angled solid black lines). Concentrations of the milk samples are expressed as REF. Data represent mean ± SD from two independent experiments (n = 4 for samples/run, n = 16 for vehicle control/run). Samples with activity below the cut-off limit, represented by the red-dotted line, were defined as bioactive. "W" denotes the week when the milk was taken. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

substances (such as antibiotics) or naturally occurring chemicals (estrogens, androgens, etc.). One has to analyze a larger number of samples in the future to draw any definitive conclusion about which biological activities in the assays that are caused by endogenous compounds and which activities that are caused by chemical pollutants. Applying *in vitro* methods could act as an early warning system to detect potentially hazardous chemicals within milk and thereby ensure the safety of milk that is needed to determine recommended actions, in a similar fashion that has been done for water samples (Lundqvist et al., 2019; Oskarsson et al., 2021). However, these methods may not be used daily, as milk has a high turnover on the market and results need to be delivered rapidly. Thus, the methods could rather be used seasonally or in a monthly fashion to investigate changes in trends.

4. Conclusions

An *in vitro*-based approach consisting of testing hormonal activities (estrogen and androgen receptors), DNA damage, oxidative stress and xenobiotic metabolism (AhR) was used to detect bioactive compounds in cow's milk. Generally, the study showed that the milk did not appear to contain detectable amounts of bioactive substances in the oxidative stress, genotoxicity and induction of estrogen/androgen receptor assays, as shown by the lack or minor response. The inhibitory mode of action on the hormonal receptor as well as AhR exerted more activity, where approximately 10–13 samples out of 32 were bioactive. Overall, no cytotoxicity was detected in three cell lines (MCF7 AREc32, TK6 and VM7Luc4E2), while nearly all milk samples at the highest REF exerted cytotoxicity in the AR-EcoScreen GR-KO M1 and the majority in DR-EcoScreen cells. The use of *in vitro* methods as a screening tool to monitor chemical hazards in milk shows great promise.

CRediT authorship contribution statement

Erica Selin: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Geeta Mandava:** Formal analysis, Investigation, Writing – review & editing. **Maria Karlsson:** Writing – review & editing, Supervision. **Johan Lundqvist:** Conceptualization, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2023.114025.

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