2018

Effects of triclosan in breast milk on the infant fecal microbiome

Candace S. Bever
University of California Davis & Foodborne Toxin Detection and Prevention Research Unit, candace.bever@ars.usda.gov

Amy A. Rand
University of California Davis & Carleton University, Ontario

Malin Nording
University of California Davis & Umeå University

Diana Taft
University of California Davis

Karen M. Kalanetra
University of California Davis

See next page for additional authors

Chemosphere 203 (2018) 467–473

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Effects of triclosan in breast milk on the infant fecal microbiome

Candace S. Bever [a, 1], Amy A. Rand [a, 2], Malin Nording [a, 3], Diana Taft [b], Karen M. Kalanetra [b, d], David A. Mills [b, d], Melissa A. Breck [b], Jennifer T. Smilowitz [b, c], J. Bruce German [b, c], Bruce D. Hammock [a, *]

[a] Department of Entomology and Nematology, and UCD Comprehensive Cancer Center, University of California Davis, Davis, CA 95616, USA
[b] Department of Food Science and Technology, University of California Davis, Davis, CA 95616, USA
[c] Foods for Health Institute, University of California Davis, Davis, CA 95616, USA
[d] Department of Viticulture and Enology, University of California Davis, Davis, CA 95616, USA

HIGHLIGHTS

- Changes in infant fecal microbiome correlates with the presence of triclosan in mother's breast milk.
- TCS is detected in breast milk from women who use triclosan-containing personal care products daily.
- The method to extract triclosan from breast milk is improved by adding salt and water.

ARTICLE INFO

Article history:
Received 4 January 2018
Received in revised form
14 March 2018
Accepted 26 March 2018
Available online 28 March 2018

Handling Editor:

Keywords:
Triclosan
Breast milk
Fecal microbiome
Infant
Personal care products

ABSTRACT

Triclosan is frequently used for its antimicrobial properties and has been detected in human serum, urine, and breast milk. Animal and molecular studies have shown that triclosan exerts a wide range of adverse health effects at both high (ppm) and low (ppb) concentrations. Since triclosan is of growing concern to human and environmental health, there is a need to improve extraction procedures and to study additional effects from triclosan exposure. In this study, we have improved triclosan extraction from breast milk by using salt ($MgSO_4$) to reduce emulsion formation and increase water polarity and water (~80%) to enhance the overall extraction efficiency (~3.5 fold). This extraction method was applied to breast milk samples collected from donors who i) recorded their use of triclosan-containing personal care products and ii) provided matching infant stool samples. Of the participants who had detectable amounts of triclosan in their breast milk, nine (75%) of them reported daily use of triclosan-containing personal care products. Levels of triclosan in breast milk were compared to the donor's infant's fecal microbiome. We found that the bacterial diversity in the fecal microbiome of the infants exposed to breast milk with detectable triclosan levels differed compared to their peers exposed to milk containing non-detectable amounts. This finding implies that exogenous chemicals are impacting microbiome diversity.

Published by Elsevier Ltd.

1. Introduction

Triclosan (TCS) is typical of a growing list of chemicals whose exposure was initially deemed insignificant but where massive commercial success lead to concerns about their impact on humans and the environment. Recent reviews on TCS recount numerous health effects ranging from endocrine-disruption to uncoupling mitochondria and interfering with ion channels (Olaniyan et al., 2016; Yueh and Tukey, 2016; Ruszkiewicz et al., 2017; Weatherly and Gosse, 2017). Most importantly, many of the cellular mechanisms are disrupted at doses around the ppm (μg mL^{-1}) level and

* Corresponding author.
E-mail addresses: candace.bever@ars.usda.gov (C.S. Bever), bdhammock@ucdavis.edu (B.D. Hammock).
[1] Present address: USDA, Agricultural Research Service, Western Regional Research Center, Foodborne Toxin Detection and Prevention Research Unit, 800 Buchanan Street, Albany, CA 94710, USA
[2] Present address: Chemistry Department, Carleton University, Ontario, Canada.
[3] Present address: Department of Chemistry, Umeå University, 901 87 Umeå, Sweden.

https://doi.org/10.1016/j.chemosphere.2018.03.186
0045-6535/Published by Elsevier Ltd.

lower. For reference, dermal exposure can be as high as 3000 μg mL^{-1} since products can be formulated to contain up to 0.3%. Although the FDA banned TCS from certain wash products (namely hand soap and body wash) in 2016 (FDA, 2016) and hospital products by the end of 2018 (FDA, 2017), it is permissible to have TCS in toothpastes, cosmetics, clothes, toys, and other products. Therefore, human exposure to TCS remains dramatically high at this time.

TCS was reported in breast milk at concentrations ranging from non-detectable to 63 ng mL^{-1} (ppb) (assuming ~3% lipids when reported per lipid weight) (Adolfsson-Erici et al., 2002; Allmyr et al., 2006a; Dayan, 2007; Ye et al., 2008; Toms et al., 2011; Wang et al., 2011; Azzouz et al., 2016). In the US, few studies have analyzed TCS in breast milk and none have correlated these levels with TCS-containing personal care product (PCP) use. A study from Sweden that demonstrated that TCS concentrations in breast milk correlated with exposure to PCPs (Allmyr et al., 2006a), with maximum levels of 1 ng mL^{-1} (ppb). Samples from the US population far exceed these values, but without identified sources of exposure (Dayan, 2007). The major fraction of TCS is eliminated from the body within 24 h of a single exposure (Sandborgh-Englund et al., 2006). However, exposure from toothpaste is usually 1–3 times per day and exposure from hand soap is typically 3–6 times per day.

TCS is frequently marketed in products for its antimicrobial properties; at low doses it is bacteriostatic and at higher doses it is bacteriocidal and active against a wide range of both Gram-negative and Gram-positive bacteria (Russell, 2004). Altered gut bacterial community structures were observed in animal models challenged via an oral route at ppm levels (Gaulke et al., 2016; Gao et al., 2017) and fish submerged in water at ppb levels (Narrowe et al., 2015). However, human studies are contradictory with a small cross-over human study failing to detect differences between time periods with and without TCS exposure (Poole et al., 2016), while another study found perturbation of adult but not infant gut microbiome in households randomly assigned to use TCS containing products compared to those not using TCS products (Ribado et al., 2017). The impact of TCS in breast milk on the infant microbiome remains unstudied.

Given that TCS was shown to modify microbiome diversity and that TCS is present in breast milk, we sought to test the hypothesis that TCS in breast milk correlates with changes in the infant microbiome. To accomplish this, we examined a cohort of US lactating women and documented their daily use of TCS-containing PCPs. We assessed women's exposure to TCS-containing PCPs by collecting survey information and by measuring TCS in their breast milk samples. We then used the results to investigate the effect on the fecal microbiome of infants whose mothers' breast milk contained TCS in detectable vs. non-detectable concentrations. We explicitly evaluated sample handling techniques with the goal of reducing the formation of emulsions, which commonly plagues human milk extraction procedures, as well as improving TCS recovery by employing a TCS ^{14}C radiotracer.

2. Materials and methods

All reagents were of analytical grade and purchased from MilliporeSigma (St. Louis, MO) or Fisher Scientific (Waltham, MA), unless otherwise noted.

2.1. Participants

Participants and their healthy term (>37 weeks gestation) infants were recruited to participate in this TCS Pilot Study from May 2013 until February 2015 from two cohorts of the one-year parent observational study, the UC Davis Lactation Study (initiated in January 2008 and ended active enrollment in February 2015) (Smilowitz et al., 2013). Briefly, participants from the larger study who expressed interest in allowing their samples to be used for further studies were re-contacted for participation in this pilot study. Those who provided informed consent to participate in this pilot, had completed the survey and provided fecal samples were included. Participants from Cohort 1 completed one recall survey and their previously collected, banked samples were analyzed. These participants were asked to recall their use of specific PCP during pregnancy and throughout the first year of lactation when they were enrolled in the parent study. Cohort 2 participants completed surveys around the time that the breast milk and infant stool samples were collected. Forty-five women (34 from Cohort 1 and 11 from Cohort 2) completed the TCS study. The UC Davis Institutional Review Board approved all aspects of the study, which was conducted in accordance with the Declaration of Helsinki regarding human experimentation, and informed consent was obtained from all subjects. This trial was registered on clinicaltrials.gov (ClinicalTrials.gov Identifier: NCT01817127).

2.2. Survey design

The survey was part of a broader metadata collection protocol that has been successfully used to examine various lactation interventions and outcomes. The specific subset of the survey devoted to TCS took advantage of the commitment and training of mothers to the broader study. The survey was comprised of an image of various TCS-containing products, listed by categories, along with its corresponding name (Figure S1). The products were identified by searching through the US Department of Health and Human Services' Household Products Database. Then, participants were asked to fill in a chart of how often a particular product was used, assessing daily (expressed as 1x/day, 2–5 x/day, or as multiple times a day), weekly (expressed as 1x/week or multiple times each week), monthly (expressed as 1x/month or a few times each month) or no use. The discrepancy in the terms was due to the changing in wording on the surveys administered to each cohort. Because of this, these terms listed were aggregated and recorded as the corresponding ‘daily/weekly/monthly’ designation.

2.3. Sample collection

A total of 45 breast milk and matched infant stool samples collected between 8 and 13 weeks postpartum were analyzed for TCS and fecal microbiome content, respectively. Details of breast milk and infant stool sample collections are reported elsewhere (Lewis et al., 2015). Breast milk samples were collected prior to the conception of this pilot project and so no special instructions were given to individuals to minimize exogenous chemical contamination.

2.4. Radioactivity studies

To determine the recovery of TCS in breast milk, ^{14}C-TCS (Moravek Biochemical, Brea, CA) was used. Samples of breast milk (1 mL) were spiked with ^{14}C-TCS (5 μL) resulting in approximately 6000 dcm per sample. Duplicate samples of each solvent type (isopropanol, acetonitrile, methanol, ethyl acetate, methyl-*tert*-butyl ether (MTBE), and hexane) were extracted in triplicate with 1 mL of solvent. Samples were inverted 30 times and then centrifuged to reduce any emulsions that formed. The distribution of radioactivity was measured by separating the extract phase from the raffinate phase. For the water-immiscible solvents, the organic extract phase was separated from the raffinate (aqueous phase often containing

precipitated proteins) and pooled. For the water-miscible solvents, the top aqueous phase was separated from the precipitated raffinate phase and pooled. Scintillator solution (15 mL) was added to each fraction to correct for quench, which accounted for the variable volumes in each fraction, and analyzed by a liquid scintillation counter (Perkin Elmer, Waltham, CA). Blank vials containing just the ^{14}C-TCS spike were measured alongside the samples to determine full recovery of the radiotracer. We further explored ^{14}C-TCS partitioning by testing for radioactivity in 0.5 mL breast milk samples spiked with ^{14}C-TCS (5 μL) and then treating the sample with i) acid (H_2SO_4, 9 M final in sample), then extracting with hexane:acetone (9:1), and ii) acetonitrile (1 mL) and adding varying amounts (in triplicate) of deionized water (0, 0.5 and 4 mL). Each sample was then extracted in triplicate with 1 mL hexane by inverting the vials 30 times. Organic extracts were pooled and the distribution of radioactivity was measured as described previously.

2.5. Sample preparation

Extraction of TCS from breast milk was completed similar to a previously described method (Allmyr et al., 2006a) with modifications based on the optimized protocol established in the radioactivity studies described in section 2.4. One notable modification included not subjecting the sample to hydrolysis to cleave any metabolic conjugates of TCS. This was omitted because previous studies had observed that conjugated species of TCS are negligible in breast milk (Ye et al., 2008; Toms et al., 2011), although present in blood and urine. Another modification was changing the derivatizing agent given the inadequate performance of the pentafluorobenzoyl derivative, which was observed to cause low yields of derivatized analyte and/or poor GC-MS peak shape (Allmyr et al., 2006b). The final modification was the incorporation of the extraction and partitioning step from the QuEChERS (quick, easy, cheap, effective, rugged, and safe) method using an organic solvent (acetonitrile) and salt ($MgSO_4$) solution (Gonzalo-Lumbreras et al., 2014).

The revised method was as follows: 10 ppm ^{13}C-TCS (5 μL, Cambridge Isotope Laboratories, Andover, MA) was spiked into each breast milk sample (2 or 3 mL). Then, acetonitrile containing 1% acetic acid (6 mL) and anhydrous $MgSO_4$ (2.1 g) were added. The samples were vortexed and then centrifuged at 3000 × g for 5 min. The supernatant was collected, water (27 mL) was added and extracted in triplicate with hexane (3 mL). Extraction involved inverting the tubes 30 times and collecting the organic fractions. To the pooled extract (9 mL), 1 M NaOH (3 mL) was added and extracted by inversion 30 times. The hexane layer was discarded. The aqueous phase was neutralized with 1 M HCl (6 mL) and extracted in triplicate with hexane (3 mL) by 30 inversions. The organic fractions were pooled and solvent was evaporated under vacuum using a ScanVac system (Neutec Group, Farmingdale, NY) until dryness. To generate the 6-point internal calibration curve, native TCS in methanol and ^{13}C-TCS were added to separate 1.5 mL plastic vials and dried under a stream of nitrogen. To the dried samples and standards, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) (50 μL) was added as a derivatization agent, heated at 37 °C for 30 min and transferred to GC vials. Detailed GC-MS parameters are given in the Supplemental Information section (S1.2).

2.6. Calibration standards and recovery experiments

To determine if the matrix had an impact on TCS detection, matrix-matched calibration curves were assembled. Donor milk from a person vigilant about avoiding known TCS-containing products was provided as the standard breast milk material. Breast milk matrix was extracted as described in section 2.5, spiked with varying concentrations of TCS (5–500 ng mL^{-1}) in triplicate, and then derivatized. Blank water samples were also spiked in triplicate with varying concentrations of TCS (n = 3).

Recovery experiments were completed to determine adequate TCS extraction efficiency. Standard breast milk material was spiked with known TCS concentrations in triplicate and then extracted and derivatized, with final theoretical TCS concentrations equaling 5, 15, 30, and 100 ng mL^{-1}.

2.7. Microbiome analysis

All infant stool samples were extracted using a ZR Fecal DNA MiniPrep kit (ZYMO, Irvine, CA, USA) and the V4 region of the 16S rRNA gene was sequenced as in Lewis et al., (2015) (Lewis et al., 2015). The resulting reads were merged using PEAR (Zhang et al., 2014), demultiplexed using FASTX tools(Lab), primers were trimmed from reads using cutadapt (Martin, 2011), and then reads were loaded into QIIME (Caporaso et al., 2010); QIIME defaults were used except where follows: operational taxonomic unit (OTU) picking was completed using the SWARM algorithm (Mahé et al., 2014), the representative sequence set was chosen using the most abundant read in each OTU, and the OTU table was filtered to remove all OTUs that only occurred in a single sample.

Only samples from infants whose mothers were enrolled in the TCS study were included in this analysis. In addition, samples with low read depth (less than 5000 reads) were excluded. After excluding low read depth samples, the sample collected closest in time to the breast milk sample used for TCS analysis was selected for inclusion. If two samples were collected within the same number of days of the TCS measurement, only the first collected sample was eligible for inclusion. Infants who received either probiotics or antibiotics prior to the collection of the included sample and all infants who had ever received formula prior to the day of included stool sample collection were excluded. After exclusions, 31 infants remained, 10 with mothers who had detectable concentrations of TCS in their milk and 21 with mothers who had non-detectable concentrations of TCS.

Before beginning analysis, OTUs without any reads in any of the included samples were excluded. Samples were rarefied to a read depth of 5000 reads. Beta diversity was visualized based on weighted, unweighted, and generalized UniFrac distance matrices using non-metric multidimensional scaling (NMDS) in R (Chen, 2012; R Core Team, 2016; Oksanen et al., 2017). NMDS was selected for ordinations because NMDS makes fewer assumptions about the underlying data structure than more common ordination methods such as principal coordinates analysis (McCune et al., 2002). The number of dimensions for the NMDS plot was chosen based on scree plots of stress (McCune et al., 2002). Differences in beta-diversity based on all three UniFrac measures between infants exposed to detectable amounts of TCS in breast milk were tested using PERMANOVA as implemented in the vegan package of R after checking for differences in dispersion, as unbalanced study design combined with differences in dispersion may skew the results of PERMANOVA (Anderson and Walsh, 2013; Oksanen et al., 2017). Differences in alpha diversity between infants from the detectable and non-detectable groups were compared using the Shannon Index and Chao1 with Kruskal-Wallis tests (Oksanen et al., 2017). The Shannon Index considers both the richness and evenness of a sample when calculating alpha diversity, while the Chao1 index considers only richness. Because of large variation in library size between samples we then used the linear discriminate analysis effect size method or LEfSE method (Segata et al., 2011) on the rarefied data to identify taxa that differed between the two groups. Only differences at the genus level and above will be discussed

Table 1
Distribution of reported types and frequency of exposures to triclosan-containing personal care products. (*For simplicity, participants with a mixture of exposure types and frequency are not presented. Only those with daily exposure to both dermal and oral products are presented).

	Dermal	Oral	Both
Daily	10	4	6
Weekly	5	0	–*
Monthly	3	3	–*
None	21	32	18

because of SWARMs tendency to split sequences means analysis at the species level may not be accurate.

3. Results and discussion

3.1. Survey findings

Exposures to TCS-containing PCPs were self-reported by each participant (Table 1). Exposure frequency was categorized as daily, weekly, monthly, or none. Exposure types were categorized as either dermal (indicated by products intended for dermal contact, such as liquid hand soaps) or oral (indicated by products intended for oral contact, such as toothpaste). Of the 45 participants, over half (27 people; 60%) reported using at least one of the TCS-containing PCPs listed in the survey. The highest frequency of using TCS-containing PCPs was for the daily use of dermal products (16; n = 10 daily dermal only + n = 6 both), followed by the daily use of oral products (10; n = 4 daily oral only + n = 6 both), which is expected given the intrinsic utility of PCPs. In all, 20 (44%) participants reported using at least one TCS-containing PCP on a daily basis, 6 of which used at least one oral and one dermal product daily.

3.2. TCS detection in breast milk

3.2.1. Radioactivity partitioning of ^{14}C-TCS

With any chemical extraction, the more steps involved, the more opportunities to reduce analyte recovery. One tool to evaluate different steps in the procedure is to use radioactively-labeled target material and detect both the extract and the raffinate portion of the sample. In our effort to select an ideal solvent, we conducted preliminary partitioning experiments using ^{14}C-TCS. By utilizing radioactivity, we were able to assess various stages of our extraction procedures without having to complete full clean-ups of the samples, nor rely on estimates of recovery, since all of the starting material is measured. Using this method, six different solvents were tested, three water-miscible (acetonitrile, isopropanol and methanol) and three water-immiscible (ethyl acetate, MTBE, hexane). The added purpose of the solvents, and in particular the miscible solvents, was to precipitate endogenous proteins from the samples. After 3 sequential extractions with the particular solvent, the extract layer for the aqueous samples retained >79% of the total TCS, while the extract layer for the immiscible solvents retained 33–82% (Fig. 1A). Interestingly, hexane, the often-used solvent for extracting lipophilic TCS from breast milk, retained the least amount of TCS at 33%. In some instances, co-solvents are added, such as water-miscible acetone at 10% (Allmyr et al., 2006b) or water-immiscible DCM at 50% (Wang et al., 2011), to change the polarity of the solution and to drive the analyte into a particular phase. So, to further explore if the addition of acid or adding acetone impacted TCS recovery into the extract phase, we tested those conditions as well, and no significant improvement was made (Fig. 1B). We then explored how adding a more polar water phase would impact the TCS recovery when hexane and acetonitrile were used as co-solvents. In this case, we saw a dramatic difference, wherein with increasing amounts of water, the recovery of TCS in the hexane extract was improved (Fig. 1C). Therefore, for the remainder of this study, we added water (up to 80%) during the first hexane extraction step.

3.2.2. TCS determination in breast milk samples and method validation

During the extraction procedure of TCS from breast milk samples, no emulsions formed in any of the samples. Emulsions are a common issue when performing extractions of milk and similar biofluids that contain lipids (Sadtler, 1909). The addition of the $MgSO_4$ salt and its ability to absorb water and increase its polarity likely reduces the amount of available water capable of emulsifying.

A matrix-matched calibration curve was completed and showed no difference with regard to the slope, nor the intercept with the y-axis. This is particularly valuable for a study like this which reduces i) the need for a valuable and sometimes harder to obtain material such as breast milk or blood, as well as, ii) reducing the likelihood of the matrix sample containing the analyte of interest to be measured.

The method recovery for the spiked samples ranged from 105 to 119% (Table S1). The largest variation is at the lowest tested level, which is expected when approaching the detection limit. The slight and consistent overestimation may be due to the presence of TCS at low levels in the donor breast milk standard, although when

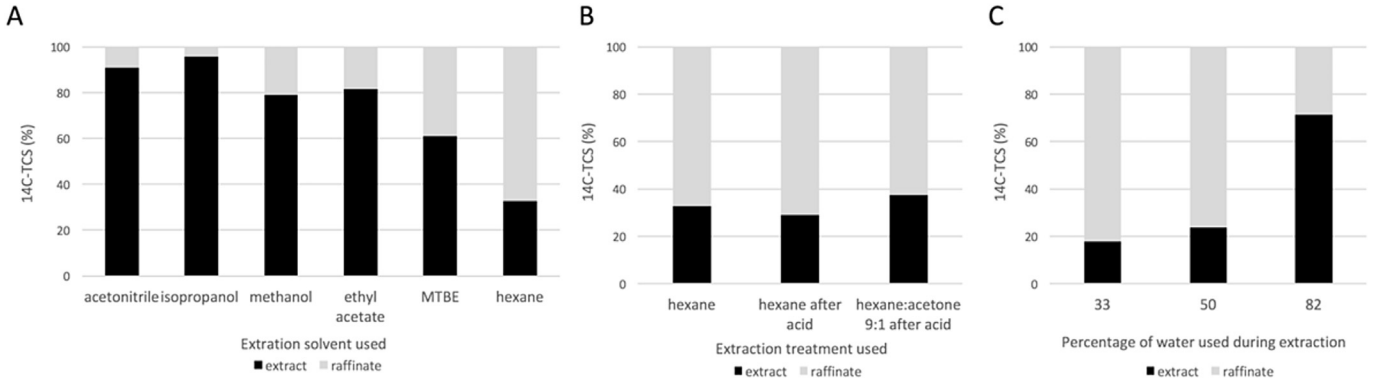

Fig. 1. Partitioning of ^{14}C-TCS in breast milk samples extracted using various solvents and methods. Each bar represents the average of duplicate samples with a standard deviation below 10%. (A) The extraction solvents varied. (B) The hexane extraction method with added reagents of acid and acetone to more precisely follow previous methods. (C) The hexane extraction method with added amounts of water.

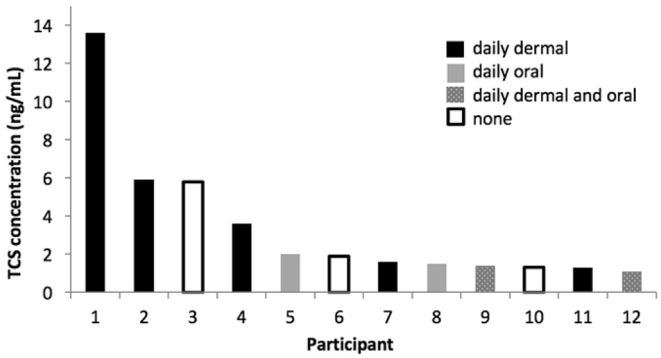

Fig. 2. Distribution of triclosan concentrations (ng mL^{-1}) in the breast milk samples (12 out of 45) with detectable amounts of TCS. The shading of each bar indicates the type and frequency of reported exposures. Only daily exposures are presented. In all, 9 of the 12 participants reported daily use of a TCS-containing PCP.

assessed as a blank without a spike, the TCS ions were below our detection limit.

The signal due to artifacts (area of the peak at *m/z* 200 and retention time 19.4 min) in eight breast milk samples with no detectable TCS was used to determine the instrument LOD and LOQ. The average signal plus 3 or 10 times the standard deviation was used to define the LOD and LOQ, respectively. This was done in order to distinguish a real signal from noise in a sample. The lowest point of the calibration curve was 25 ppb TCS on column, which corresponded to 0.4 ng mL^{-1} breast milk. This determined the lowest quantifiable amount in breast milk, and therefore corresponded to the method LOQ. All samples with signals above the instrument LOD contained TCS levels above 0.4 ng mL^{-1} breast milk (Fig. 3). Four of these (breast milk samples from participants 1–4) produced signals also above the instrument LOQ.

For comparison to previously published methods for TCS quantification in breast milk, we report improved LOQ in contrast to Ye et al. (1 ng mL^{-1} with HPLC-MS/MS) (Ye et al., 2008), but not as good as Allmyr et al. (0.018 ng g^{-1} with GC-MS) (Allmyr et al., 2006b).

3.2.3. TCS detection and reported TCS-containing PCP use

Of the 45 samples analyzed, 12 (27%) of them produced signals above the method LOD (Fig. 2), ranging from 1 to 13.6 ng mL^{-1} (ppb). These 12 are referred to as the 'detectable amounts of TCS in breast milk group', while the remaining 33 are referred to as the 'non-detectable amounts of TCS in breast milk group' for the purposes of evaluating the infant fecal microbiome analyses. Of the 12 with detectable TCS levels, 75% (n = 9) reported daily exposure to at least one TCS-containing PCP intended for either dermal or oral contact. More specifically, daily dermal exposure to a TCS-containing PCP was reported for 58% (n = 7), while daily oral exposure was reported for 33% (n = 4). Only two people (17%) indicated daily exposure to both dermal and oral products (Table 2). Given that Cohort 2 was a year after Cohort 1, and that only one person (9%) from Cohort 2 (n = 11) had detectable amounts of TCS compared to 12 people (32%) from Cohort 1 who had detectable amounts of TCS, this suggests that exposure nationally is declining as expected given the 2016 FDA guidelines for TCS (FDA, 2016), possibly related to more informed choices, public awareness of TCS and its potential harm, or less availability of TCS containing products. Comparing the detectable and non-detectable groups, Table 2 shows the distribution of daily exposure for dermal, oral and dermal + oral products. The distributions of those using products daily are very similar (i.e., 11 from the non-detectable group, comprised from 5 dermal, 2 oral, and 4 dermal + oral; and 9 from the detectable group, comprised of 5 dermal, 2 oral, and 2 dermal + oral), however there are many more participants total in the non-detecable group. Thus, only 33% (11 out of 33) report using TCS-containing PCPs on a daily basis in the non-detectable group, compared to 75% (9 out of 12) from the detectable group.

Limitations to this study include the small sample size (n = 45) and the reliance of survey results on participant recall of PCP use

Table 2
Distributions of reported daily exposure to TCS-containing PCPs separated by participants who had detectable and non-detectable amounts of TCS in their breast milk samples.

	Detected	Non-detected
No daily use	3	22
Dermal use only	5	5
Oral use only	2	2
Dermal and oral use	2	4
TOTAL	12	33

Fig. 3. Boxplots of alpha diversity. By the Shannon Index, the group exposed to non-detectable levels of TCS in breast milk had significantly greater alpha diversity than the group exposed to detectable levels of TCS in breast milk group. There were no significant differences in alpha diversity by the Chao1 index.

and without additional confirmation. The survey was designed to avoid bias for or against the use of antibacterial products, although, as with all studies using human subjects, knowledge on this subject matter may have been obtained through media news and marketing.

This study documents that mothers who use commercial products that contain TCS, are more likely to have higher levels of TCS in their breast milk than those who identify and use products that do not contain TCS. The strength of this correlation suggests that other known exposures from materials coated or impregnated with TCS, such as some kitchenwares, specialized clothing, or certain office products, while they may cumulatively result in elevated body burden levels, the evidence suggests that their contributions are minor.

3.3. Infant fecal microbiome

Infant stool samples from the time point closest to the breast milk sample used for TCS analysis were assessed for differences in alpha diversity, beta diversity, and differential abundance in taxa between infants fed milk containing detectable levels of TCS and non-detectable levels of TCS. The unweighted UniFrac and generalized UniFrac (Lozupone and Knight, 2005) NMDS plots used 3 dimensions, the weighted UniFrac NMDS plot needed only 2 dimensions (Figure S2). There was no visible separation between detectable and non-detectable stool samples on any plot. The dispersion of samples between the two groups was significantly different using both unweighted UniFrac ($p = 0.036$) and generalized UniFrac ($p = 0.0074$), and was borderline significant using weighted UniFrac ($p = 0.054$). There was no significant difference using PERMANOVA by any of the UniFrac measures.

In the alpha diversity analysis, the stool samples from infants fed breast milk with detectable levels of TCS had significantly lower alpha diversity by the Shannon Index ($p = 0.025$) but the Chao1 index did not differ significantly ($p = 0.97$) (Fig. 3). This suggests that while exposure to breast milk containing detectable levels of TCS did not reduce the richness (total number of OTUs) found in the infant gut, it did result in a reduction in evenness. This suggests that there are greater differences in the distribution of the relative abundances of OTUs in the detectable group than the non-detectable group.

LEfSE also identified several differential abundant taxa at the genus level and above (Fig. 4). The infants exposed to non-detectable levels of TCS in breast milk had significantly higher relative abundance of family Lachnospiraceae, class Erysipelotrichi, order Erysipelotrichales, and family Erysipelotrichaceae. The infants exposed to detectable levels of TCS in breast milk had significantly higher relative abundance of genus *Dermabacter*, order Rhodospirillales, and family Rhodospirillaceae. While variations in abundant taxa have been seen in fish exposed to TCS, the microbiome response to TCS in fish differed from what was observed in this study. The TCS fish exhibited increased Shannon Index values with higher triclosan exposure and also showed significant differences between groups by PERMANOVA (Narrowe et al., 2015), where this study found decreased Shannon Index values with TCS exposure and no significant differences using PERMANOVA.

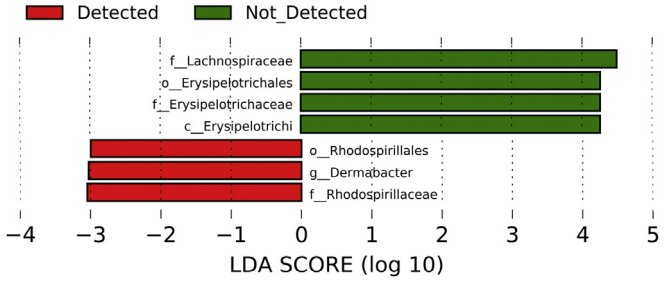

Fig. 4. LEfSE results comparing infants exposed to detectable and non-detectable levels of TCS in breast milk. Taxa in green were significantly enriched in infants exposed to non-detectable levels of TCS in breast milk and taxa in red were significantly enriched in infants exposed to detectable levels of TCS in breast milk. OTU level differences and unidentified taxa are excluded from the plot. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Overall, the purpose of this pilot study was to gain a preliminary appreciation of the connectivity between TCS exposure from breast milk and subsequent infant fecal microbiome diversity. We recognize that TCS is not the only exogenous chemical in breast milk (LaKind et al., 2004) and that endogenous components are also variable and influence the infant fecal microbiome (Ballard and Morrow, 2013). Nonetheless, although this study was a single location with a small sample size ($n = 12$), the evidence of an altered fecal microbiome along with TCS detected in the breast milk fed to that individual is larger than the inherent variability that might have been observed by chance alone. Furthermore, the functional consequences of the changes in the infant gut microbiome described here remain unclear. For example, while infants are known to be colonized with Lachnospiraceae, the role of Lachnospiraceae in the infant gut remains unclear (Sagheddu et al., 2016).

This study examined infants ranging from 8 to 13 weeks (~2–3 months old) who were fed exclusively breast milk. Ingestion of TCS from breast milk may not be the only source of exposure to an infant. Chemicals could be transferred to the infant via sucking on hands, pacifiers, bottle nipples, and clothing/fabrics (Ginsberg and Balk, 2016). In this study, we did not include dishwashing liquids, of which a few formulations do contain TCS and could have left residues on pacifiers and bottles (Tsai et al., 2008). If breast pump parts used to collect milk for this study were washed similarly, the contaminating TCS might have appeared in the breast milk analysis, and if it were present on bottles used for the infant, would still have resulted in exposure to the infant. Due of the rapid nature of TCS removal from the body, an ideal follow-up study would be to request mothers using TCS-containing PCPs to stop use for a set amount of time and to observe the changes in their breast milk concentrations and the impact (potentially short-term or long-term) on their infant's fecal microbiome.

4. Conclusion

The developing infant microbiome is particularly sensitive to modulation from environmental factors including diet, mode of delivery and antibiotic use, among others (Blaser and Dominguez-Bello, 2016). The outcomes of this study document that the bacterial diversity in the fecal microbiome of the infants exposed to breast milk containing detectable levels of TCS differed compared to their peers who were exposed to breast milk containing non-detectable levels of TCS. This study demonstrates that exogenous chemicals are correlated with altering microbiome diversity in the early developing infant intestinal community. Human health is increasingly associated with the successful development of the microbial communities within humans and more specifically on microbiome functionality. Given the increasingly important role the establishment of a healthy infant gut microbiome has to healthy phenotypes later in life (Charbonneau et al., 2016), understanding how environmentally-acquired antimicrobials such as TCS influence infant gut development is critical to identify and rectify problematic shifts in infant gut health. This study demonstrated that reported daily use of TCS-containing PCPs correlates with

measured exposure levels. The implications of these exposures to microbial communities should now be considered when developing, marketing, and regulating chemicals that alter the microbial communities important to human health. Additional findings from this work deliver better methods for extracting TCS including i) increasing salt to eliminate emulsion formation and ii) increasing water as a solvent to drive more TCS into the hexane extract.

Acknowledgements

We are grateful for the mothers and their infants for participating in this project. This study was supported by UC Davis Environmental Health Sciences Core Center P30 ES02351, UC Davis Superfund Research Program P42 ES04699, and National Institutes of Health awards AT007079 and AT008759.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2018.03.186.

References

Adolfsson-Erici, M., Pettersson, M., Parkkonen, J., Sturve, J., 2002. Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. Chemosphere 46, 1485–1489.
Allmyr, M., Adolfsson-Erici, M., McLachlan, M.S., Sandborgh-Englund, G., 2006a. Triclosan in plasma and milk from Swedish nursing mothers and their exposure via personal care products. Sci. Total Environ. 372, 87–93.
Allmyr, M., McLachlan, M.S., Sandborgh-Englund, G., Adolfsson-Erici, M., 2006b. Determination of triclosan as its pentafluorobenzoyl ester in human plasma and milk using electron capture negative ionization mass spectrometry. Anal. Chem. 78, 6542–6546.
Anderson, M.J., Walsh, D.C., 2013. PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: what null hypothesis are you testing? Ecol. Monogr. 83, 557–574.
Azzouz, A., Rascon, A.J., Ballesteros, E., 2016. Simultaneous determination of parabens, alkylphenols, phenylphenols, bisphenol A and triclosan in human urine, blood and breast milk by continuous solid-phase extraction and gas chromatography-mass spectrometry. J. Pharmaceut. Biomed. Anal. 119, 16–26.
Ballard, O., Morrow, A.L., 2013. Human milk composition: nutrients and bioactive factors. Pediatr. Clin. 60, 49–74.
Blaser, M.J., Dominguez-Bello, M.G., 2016. The human microbiome before birth. Cell Host Microbe 20, 558–560.
Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Br. J. Pharmacol. 7.
Charbonneau, M.R., Blanton, L.V., DiGiulio, D.B., Relman, D.A., Lebrilla, C.B., Mills, D.A., Gordon, J.I., 2016. A microbial perspective of human developmental biology. Nature 535, 48–55.
Chen, J., 2012. GUniFrac: Generalized UniFrac Distances.
Dayan, A.D., 2007. Risk assessment of triclosan [Irgasan] in human breast milk. Food Chem. Toxicol. 45, 125–129.
FDA, 2016. FDA Issues Final Rule on Safety and Effectiveness of Antibacterial Soaps:. FDA News Release. US DHHS.
FDA, 2017. Safety and Effectiveness for Health Care Antiseptics; Topical Antimicrobial Drug Products for Over-the-counter Human Use. DHHS.
Gao, B., Tu, P., Bian, X., Chi, L., Ru, H., Lu, K., 2017. Profound perturbation induced by triclosan exposure in mouse gut microbiome: a less resilient microbial community with elevated antibiotic and metal resistomes. BMC Pharmacol Toxicol 18, 46.
Gaulke, C.A., Barton, C.L., Proffitt, S., Tanguay, R.L., Sharpton, T.J., 2016. Triclosan exposure is associated with rapid restructuring of the microbiome in adult zebrafish. PLoS One 11, e0154632.
Ginsberg, G.L., Balk, S.J., 2016. Consumer products as sources of chemical exposures to children: case study of triclosan. Curr. Opin. Pediatr. 28, 235–242.
Gonzalo-Lumbreras, R., Sanz-Landaluze, J., Camara, C., 2014. Analytical performance of two miniaturised extraction methods for triclosan and methyltriclosan, in fish roe and surimi samples. Food Chem. 146, 141–148.
Lab, H., FASTX Toolkit.
LaKind, J.S., Wilkins, A.A., Berlin, C.M., 2004. Environmental chemicals in human milk: a review of levels, infant exposures and health, and guidance for future research. Toxicol. Appl. Pharmacol. 198, 184–208.
Lewis, Z.T., Totten, S.M., Smilowitz, J.T., Popovic, M., Parker, E., Lemay, D.G., Van Tassell, M.L., Miller, M.J., Jin, Y.-S., German, J.B., 2015. Maternal fucosyltransferase 2 status affects the gut bifidobacterial communities of breastfed infants. Microbiome 3, 13.
Lozupone, C., Knight, R., 2005. UniFrac: a new phylogenetic method for comparing microbial communities. Appl. Environ. Microbiol. 71, 8228–8235.
Mahé, F., Rognes, T., Quince, C., de Vargas, C., Dunthorn, M., 2014. Swarm: robust and fast clustering method for amplicon-based studies. PeerJ 2, e593.
Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet. journal 17, 10–12.
McCune, B., Grace, J.B., Urban, D.L., 2002. Analysis of Ecological Communities. MjM Software Design, Gleneden Beach, OR.
Narrowe, A.B., Albuthi-Lantz, M., Smith, E.P., Bower, K.J., Roane, T.M., Vajda, A.M., Miller, C.S., 2015. Perturbation and restoration of the fathead minnow gut microbiome after low-level triclosan exposure. Microbiome 3, 6.
Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2017. Vegan: Community Ecology Package.
Olaniyan, L.W., Mkwetshana, N., Okoh, A.I., 2016. Triclosan in water, implications for human and environmental health. SpringerPlus 5, 1639.
Poole, A.C., Pischel, L., Ley, C., Suh, G., Goodrich, J.K., Haggerty, T.D., Ley, R.E., Parsonnet, J., 2016. Crossover control study of the effect of personal care products containing triclosan on the microbiome. mSphere 1.
R Core Team, 2016. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Ribado, J.V., Ley, C., Haggerty, T.D., Tkachenko, E., Bhatt, A.S., Parsonnet, J., 2017. Household triclosan and triclocarban exposure impacts the adult intestinal microbiome but not the infant intestinal microbiome. bioRxiv 126334.
Russell, A.D., 2004. Whither triclosan? J. Antimicrob. Chemother. 53, 693–695.
Ruszkiewicz, J.A., Li, S., Rodriguez, M.B., Aschner, M., 2017. Is Triclosan a neurotoxic agent? J. Toxicol. Environ. Health B Crit. Rev. 20, 104–117.
Sadtler, S.S., 1909. Method of avoiding emulsions in organic analysis. J. Ind. Eng. Chem.-us 1, 479–480.
Sagheddu, V., Patrone, V., Miragoli, F., Puglisi, E., Morelli, L., 2016. Infant early gut colonization by Lachnospiraceae: high frequency of ruminococcus gnavus. Front Pediatr 4, 57.
Sandborgh-Englund, G., Adolfsson-Erici, M., Odham, G., Ekstrand, J., 2006. Pharmacokinetics of triclosan following oral ingestion in humans. J. Toxicol. Environ. Health 69, 1861–1873.
Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., Huttenhower, C., 2011. Metagenomic biomarker discovery and explanation. Genome Biol. 12, R60.
Smilowitz, J.T., O'Sullivan, A., Barile, D., German, J.B., Lonnerdal, B., Slupsky, C.M., 2013. The human milk metabolome reveals diverse oligosaccharide profiles. J. Nutr. 143, 1709–1718.
Toms, L.M., Allmyr, M., Mueller, J.F., Adolfsson-Erici, M., McLachlan, M., Murby, J., Harden, F.A., 2011. Triclosan in individual human milk samples from Australia. Chemosphere 85, 1682–1686.
Tsai, S.W., Shih, M.W., Pan, Y.P., 2008. Determinations and residual characteristics of triclosan in household food detergents of Taiwan. Chemosphere 72, 1250–1255.
Wang, H., Zhang, J., Gao, F., Yang, Y., Duan, H., Wu, Y., Berset, J.D., Shao, B., 2011. Simultaneous analysis of synthetic musks and triclosan in human breast milk by gas chromatography tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 879, 1861–1869.
Weatherly, L.M., Gosse, J.A., 2017. Triclosan exposure, transformation, and human health effects. J. Toxicol. Environ. Health B Crit. Rev. 20, 447–469.
Ye, X., Bishop, A.M., Needham, L.L., Calafat, A.M., 2008. Automated on-line column-switching HPLC-MS/MS method with peak focusing for measuring parabens, triclosan, and other environmental phenols in human milk. Anal. Chim. Acta 622, 150–156.
Yueh, M.F., Tukey, R.H., 2016. Triclosan: a widespread environmental toxicant with many biological effects. Annu. Rev. Pharmacol. Toxicol. 56, 251–272.
Zhang, J., Kobert, K., Flouri, T., Stamatakis, A., 2014. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics 30, 614–620.