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Epidemiology of Allergic Disease

Association of bacterial load in drinking water and allergic diseases in childhood

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Abstract

Background: Treatment of drinking water may decrease microbial exposure.

Objective: To investigate whether bacterial load in drinking water is associated with altered risk of allergic diseases.

Methods: We recruited 1,110 schoolchildren aged 6-16 years between 2011 and 2013 in Požega-Slavonia County in Croatia, where we capitalized on a natural experiment whereby individuals receive drinking water through public mains supply or individual wells. We obtained data on microbial content of drinking water for all participants; 585 children were randomly selected for more detailed assessments, including skin prick testing. Since water supply was highly correlated with rural residence, we compared clinical outcomes across four groups (Rural/Individual, Rural/Public, Urban/Individual and Urban/Public). For each child, we derived quantitative index of microbial exposure (bacterial load in the drinking water measured during the child's first year of life).

Results: Cumulative bacterial load in drinking water was higher (median [IQR]: 6390 [4190-9550] vs 0 [0-0]; $P < .0001$), and lifetime prevalence of allergic diseases was significantly lower among children with individual supply (5.5% vs 2.3%, $P = .01$; 14.4% vs 6.7%, $P < .001$; 25.2% vs 15.1%, $P < .001$; asthma, atopic dermatitis [AD] and rhinitis, respectively). Compared with the reference group (Urban/Public), there was a significant reduction in the risk of ever asthma, AD and rhinitis amongst rural children with individual supply: OR [95% CI]: 0.14 [0.03,0.67], $P = .013$; 0.20 [0.09,0.43], $P < .001$; 0.17 [0.10,0.32], $P < .001$. Protection was also observed in the Rural/Public group, but the effect was consistently highest among Rural/Individual children. In the quantitative analysis, the risk of allergic diseases decreased significantly with increasing bacterial load in drinking water in the first year of life (0.79 [0.70,0.88], $P < .001$; 0.90 [0.83,0.99], $P = .025$; 0.80 [0.74,0.86], $P < .001$; current wheeze, AD and rhinitis).

Conclusions and Clinical Relevance: High commensal bacterial content in drinking water may protect against allergic diseases.

KEYWORDS

atopy, bacterial load, children, drinking water, microbiota, well water

Turkalj, Drkulec and Haider are equal contribution, joint first authors.

Nogalo and Custovic are equal contribution, joint senior authors.

1 | INTRODUCTION

Studies from different parts of the world have shown that allergic diseases are less common in rural compared to urban areas.¹ However, data from countries in transition suggest that rather than urban living per se, it is affluence and westernized lifestyle that are associated with higher risk.²⁻⁴ The key role of specific protective environmental exposures has been highlighted in studies of children in traditional farming families, which have shown markedly reduced prevalence of asthma and sensitization compared to control rural populations.^{5,6} The strongest protective effect was observed for the contact with farm animals and intake of unprocessed farm milk,⁷ particularly among genetically susceptible individuals.⁸ Both of these protective features are associated with high microbial exposures, and the effect of unprocessed milk consumption is explained partly by the absence of heating.⁹

Further studies capitalized on “natural experiments” which enabled comparisons of genetically similar populations with different lifestyles and/or living conditions, and confirmed marked differences in asthma prevalence between Amish and Hutterite schoolchildren,¹⁰ or populations in Finnish and Russian Karelia.¹¹ These studies offer insights into potential mechanisms of protection, such as the finding that Amish environment protects against asthma by engaging and modulating innate immunity.¹⁰ In an experimental murine model, intranasal exposure of pregnant mice to extracts of one of the main microbial constituents of farm dust (*Acinetobacter lwoffii*) protected against asthma development in offspring,¹² and the demonstration that this process is TLR-dependent suggests that direct sensing of the protective microbial stimuli by the maternal innate immune system is important.¹² The evidence to date is consistent with the notion that microbial diversity is a hallmark of farm homes and associated with reduced risk of asthma, but importantly a farm-like microbial compositional structure in non-farm homes is also associated with protection.¹³

Modern treatment of drinking water decreased exposure to pathogens, but also altered the exposure to commensal bacterial strains, and it is possible that altered microbial content in drinking water may impact upon gut microbiota and the development of immune responses. A study comparing Finnish and Russian Karelia suggested that high microbial content in drinking water in Russian Karelia may be associated with a reduced risk of allergic sensitization, independently from other putative protective factors.¹⁴ However, although the observed relationship was dose-dependent and biologically plausible, due to the marked economic gap between the areas, the living conditions in Finnish Karelia are very different from those in Russian Karelia, and it is difficult to infer causality on any single factor.¹⁴

We tested the hypothesis that bacterial load in drinking water is associated with altered risk of allergic diseases by taking advantage of a natural experiment in a unique area of Eastern Croatia, in which individuals receive drinking water through different supply systems (either public supply or individual wells). The type of water supply is determined by external factors (the development of the local water supply system), thereby resembling the experimental and control conditions. Microbiological analysis of drinking water has been carried out regularly for the last three decades. The population is genetically

uniform with otherwise similar lifestyle, cultural background and living conditions, offering an opportunity to test our hypothesis.

2 | METHODS

2.1 | Study design, setting and participants

In the Phase 1 of the study, we recruited 1110 school children aged 6 to 16 years from a random sample of 12 schools (one large urban school and 11 small rural schools) in Požega-Slavonia County in Eastern Croatia (Figure S1). Schools were supplied with written material containing the information about the study. Teachers, parents and children were given oral and written information about the study at dedicated parents/teacher meetings, and a written informed consent from the parents/legal guardians was obtained.

Following the completion of the survey, it became evident that some children attending the urban school were living in the surrounding rural areas and travelled to school daily. We therefore proceeded to the phase 2, in which approximately half of the study participants (N = 541) were randomly selected for a more detailed assessment, which included skin testing and further questionnaires (including questions on area of residence and farming). Studies were carried out between 2011 and 2013. Ethical approvals were granted by the Ethic Committees of the Children's Hospital Srebrnjak (Zagreb) and the County Hospital Požega.

2.2 | Data sources/measurement

2.2.1 | Phase 1

A validated ISAAC phase 1 core questionnaire¹⁵ was distributed by teachers to collect information on parentally reported symptoms, physician-diagnosed illnesses and medication use. We also collected data on the drinking water supply (public mains and/or individual wells). Questionnaires were completed at home by parents and returned to the school.

2.2.2 | Phase 2

We used a questionnaire to collect data about home location (urban or rural area), farming practices and socio-economic status (SES, including parental education, employment status and family income). Allergic sensitization was ascertained using skin prick tests (SPT) against 9 inhalant allergens: *D pteronyssinus*, *D farinae*, cockroach, *Ambrosia elatior*, grass mix, birch, hazel, *Cladosporium* and *Alternaria* (ALK, Denmark).

2.3 | Analysis of microbial content of drinking water

We obtained data on the microbial content of drinking water for the period 1997-2007 to capture the early-life exposure for all study

participants (who were born during this period). Water samples were collected at random points from the public water distribution network and from each well in households with individual water supply. Samples were analysed by the Department of Health Ecology, Institute of Public Health, Požega-Slavonia County. Physicochemical and microbiological quality of drinking water was determined using standardized methodology recommended by the Croatian national centre (Institute of Public Health Dr Andrija Štampar, Zagreb) and according to methods described in the national Drinking Water Safety Regulation (for details see Tables S1-S3). Microbial content included quantification of *Clostridium perfringens*, *Pseudomonas aeruginosa*, *E coli* and other coliform bacteria. We first compared the overall bacterial load over the entire collection period (a sum of bacterial colony-forming units—CFUs) between populations receiving drinking water through public mains supply or individual wells. For each participant, we then derived an individual quantitative index of exposure based on the bacterial content of drinking water measured during the child's first year of life. We also calculated quantitative exposure in each child's fifth year of life.

2.4 | Definition of outcomes

2.4.1 | Asthma ever

Positive answer to the question “Has your child ever had asthma?” or “Physician diagnosed asthma ever.”

2.4.2 | Current wheeze

Positive answer to the question “Has your child had wheezing or whistling in the chest in the last 12 months?”

2.4.3 | Atopic dermatitis (AD) ever

Positive answer to “Has your child ever had an itchy rash which was coming and going for at least 6 months?” or “Has your child ever had eczema?”

2.4.4 | Current AD

Positive answer to “Has your child had an itchy rash that comes and goes in the last 12 months?”

2.4.5 | Rhinitis ever

Positive answer to “Has your child ever had a problem with sneezing or a runny or blocked nose when he/she DID NOT have a cold of flu?”

2.4.6 | Current rhinitis

Positive answer to “In the past 12 months, has your child had a problem with sneezing or a runny or blocked nose when he/she did not have a cold or the flu?”

2.4.7 | Allergic sensitization

SPT mean weal diameter 3 mm greater than negative control to at least one allergen.

2.5 | Statistical analysis

Statistical analysis was performed using Stata Statistical Software, Release 15. Basic descriptive summaries of data were obtained, and differences between investigated groups were calculated using the chi-square test for categorical data, and the Wilcoxon rank sum test for quantitative variables. To test if the type of water supply (public vs individual) was associated with health outcomes, we ran multivariable logistic regression models. As the majority of individual wells in our study population were in rural areas, we ran additional multivariable regression models which included interactions between water supply type and location. We adjusted all models with confounding variables including age, sex and SES (defined as paternal employment status). Results are reported as odds ratios (OR) with 95% confidence intervals (CIs).

3 | RESULTS

A total of 1110 children years were recruited for the phase 1 of the study (6-10 years, N = 551; 11-16 years, N = 559). We excluded 80 children, as data on the type of water supply were missing. A further 52 were excluded for having a mixed water supply; hence, our sample comprised 978 children, of whom 474 (48.5%) attended rural schools, and 504 (51.5%) were from the urban school. Of those, 494 accessed drinking water through the public mains water supply, and 484 through individual wells. There was a marked and highly significant difference in the cumulative bacterial load in drinking water, with samples from individual wells having a much higher bacterial load (median [IQR]: individual 6390 [4190-9550] vs public 0 [0-0], $P < .0001$, Table 1).

Demographic characteristics and clinical outcomes in the whole population and by water supply type are presented in Table 1. Among 484 children with individual water supply, the majority (91.3%) were attending rural schools. Gender distribution did not significantly differ between different types of water supply. However, there was a significant difference in age, with younger children (age 6-10) predominantly using individual (86.2%) compared with public water supply (21.9%, $P < .0001$).

3.1 | Association of bacterial load in drinking water and clinical outcomes

Estimated rates for clinical outcomes were significantly lower among children with the individual drinking water supply for lifetime prevalence of asthma, AD and rhinitis (5.5% vs 2.3%, $P = .011$; 14.4% vs 6.7%, $P < .001$; and 25.2% vs 15.1%, $P < .001$, respectively; Table 1). Current wheeze, AD and rhinitis were also significantly less common among children living in homes with individual wells (Table 1).

These descriptive analyses suggested that health outcomes may differ by the type of water supply. However, individual wells water supply was also highly correlated with attending rural schools, and in Phase 1 of the study, we did not have sufficient information to accurately ascertain the area of residence or farming practices of the participating families. We therefore proceeded to the Phase 2, in which we collected additional more accurate information about the home location, farming practices and SES in 585 participants, 532 of whom gave consent for the objective assessment of allergic sensitization. Farming was indistinguishable from rural residence, and we used urban/rural residence as a proxy in further analyses.

Summary descriptive statistics for each of the clinical outcomes across the four groups (Rural/Individual, Rural/Public, Urban/Individual and Urban/Public) is shown in Table 2. Outcomes differed significantly,

with the lowest proportion of children with asthma, wheeze, AD and rhinitis observed among children living in rural areas who had individual wells water supply. To differentiate between the effects of location/farming and water supply, we ran models which included terms for the interaction of the two variables, using children receiving drinking water through public mains supply and living in an urban location as a reference. Results are presented in Table 3. Compared to urban children receiving water through public supply, there was a significant reduction in the lifetime risk of all outcomes amongst those living in rural area and using individual water supply: OR [95% CI]: 0.14 [0.03,0.67], $P = .013$; 0.42 [0.25,0.72], $P = .001$; 0.20 [0.09,0.43], $P < .001$; 0.17 [0.10,0.32], $P < .001$; ever asthma, wheeze, AD and rhinitis. The risk of current symptoms was also significantly lower in this group. Reduction in risk was observed among rural children who were receiving drinking water through the public supply, but the proportion of children with asthma ever, and current and ever wheeze, AD and rhinitis was consistently lower among those living in rural areas who were using water from individual wells. The risk of sensitization was lower in rural children, with no significant difference according to water supply.

We then investigated whether there was a dose-response relationship between the quantitative index of exposure (derived as the bacterial content in the drinking water in each child's first year of life). Results are presented in Table 4. In a multivariable logistic

	Whole sample (n = 978)	Public (n = 494)	Individual (n = 484)	P-value
Cumulative bacterial load in drinking water (CFU/mL); median (IQR)				
Total bacterial load	0 (0-5860)	0 (0-0)	6390 (4190-9550)	<.001
Coliform	0 (0-1860)	0 (0-0)	1970 (1370-3460)	<.001
<i>Clostridium perfringens</i>	0 (0-620)	0 (0-0)	670 (230-860)	<.001
<i>Pseudomonas aeruginosa</i>	0 (0-2270)	0 (0-0)	2470 (1580-6180)	<.001
Child characteristics: n (%)				
Gender (Male)	501/978 (51.2)	258 (52.2)	243 (50.2)	.527
Age (6-10)	525/978 (53.7)	108 (21.9)	417 (86.2)	<.001
(11-16)	453/978 (46.3)	386 (78.1)	67 (13.8)	
Rural schools	474/978 (48.5)	32 (6.5)	442 (91.3)	<.001
Urban school	504/978 (51.5)	462 (93.5)	42 (8.7)	
Lifetime prevalence of symptoms: n (%)				
Asthma ever	38/973 (3.9)	27 (5.5)	11 (2.3)	.011
Wheeze ever	195/973 (20.0)	108 (22.0)	87 (18.1)	.132
Atopic dermatitis ever	103/973 (10.6)	71 (14.4)	32 (6.7)	<.001
Rhinitis ever	195/965 (20.2)	123 (25.2)	72 (15.1)	<.001
Current symptoms: n (%)				
Wheeze	102/970 (10.5)	66 (13.4)	36 (7.5)	0.003
Atopic dermatitis	69/965 (7.2)	43 (8.8)	26 (5.5)	.043
Rhinitis	148/965 (15.34)	86 (17.6)	62 (13.0)	.049

TABLE 1 Demographic characteristics of the study population

regression model adjusted for age, sex and SES, the risk of life-time asthma, wheeze, AD and rhinitis, and of current wheeze, AD and rhinitis decreased significantly with increasing bacterial load in drinking water (eg OR [95% CI]: 0.77 [0.62,0.95], $P = .016$ and 0.79 [0.70,0.88], $P < .001$, asthma ever and current wheeze, respectively).

Additional sub-analyses among 188 children with individual water supply confirmed the dose-response relationship between bacterial content in the drinking water in the first year of life and the risk of wheezing (OR [95% CI]: 0.61 [0.40-0.93], $P = .021$); however, there was no association between bacterial content in each child's fifth year of life and any of the outcomes (Table S4).

4 | DISCUSSION

4.1 | Main findings

Results of our study suggest that high microbial content in drinking water may be associated with a reduced risk of allergic diseases in childhood. We assessed quality of drinking water from homes of the study participants and have capitalized on the availability of quantitative data on microbial content of water during each child's first year of life. The dose-response relationship (in that the risk of allergic diseases decreased significantly with increasing bacterial load in drinking water) further strengthens the findings and suggests that the observed associations may be specific to microbial content of drinking water and are not a surrogate marker for other yet unidentified exposures. However, we wish to emphasize that the results we report are associations, and we cannot infer causality.

4.1.1 | Limitations

We acknowledge a number of limitations to our study. Firstly, the number of children included in some of the analyses was small (eg only 21 children in the Phase 2 were living in an urban area and using individual water supply). The water supply was highly correlated with the area of residence, and ~90% of individual wells in our sample were in rural areas. Given the development of the water supply system from more urbanized towards more rural areas, it is not surprising that there was a preponderance of wells in the rural population. However, these limitations make it difficult to distinguish with certainty whether water supply type had additional effects over and above the rural location (or some other unmeasured exposure). Although our results suggested that the strongest protective effect was amongst children living in rural area who were using individual water supply, given the small sample size of the urban/individual group, great caution is needed in interpreting results. However, the dose-response relationship between bacterial load in drinking water and the risk of allergic phenotypes provides some evidence that the observed effects may be due to the microbial content.

During the conduct of the study, it became clear that our strategy to use school location as a proxy of urban/rural living (as done in

a number of previous studies^{2-4,16,17}) was not optimal for this study area, as some children from urban school were living in the surrounding, more rural areas. Furthermore, we did not have enough information to accurately ascertain farming practices of the participating families. We therefore completed Phase 2, in which we collected additional accurate information about the home location (urban or rural area), farming practices and socio-economic status, and carried out the objective assessment of allergic sensitization. However, due to the constraints related to funding and personnel, we could only invite half of the original participants for further assessment. We could not differentiate between rural location and farming, because almost all participants who were living in rural areas also reported some farming practices. It is of note that farming in this part of the country is almost exclusively small-hold, supporting a single family, with a mixture of subsistence crop and pig and poultry farming, with few dairy and cattle farms. It is also of note that there are no major urban centres in the County (its population was 78,034 as of the 2011 census).

We acknowledge that we did not collect information on all potentially important pre- and perinatal factors and environmental exposures, including mode of delivery and air pollution. It is well established that air pollution contributes to worsening asthma control and increases the risk of asthma exacerbations. Furthermore, perinatal exposure to ambient ultrafine particles (<0.1 mm diameter) and maternal exposure to traffic-related NO₂ during pregnancy have been linked to the onset of asthma in children.^{18,19} It is of note that we observed differences in allergic diseases between rural children using wells or mains water supply, with a dose-response relationship between bacterial content in drinking water and the risk of allergies. These data suggest that it is unlikely that differences in important unmeasured factors which differ between urban and rural children (such as air pollution or health-seeking behaviour) had major impact on our results.

Another limitation of our study is that although the measurement of the microbial content of drinking water included quantification of *Clostridium perfringens*, *Pseudomonas aeruginosa*, *E coli* and other coliform bacteria, we could not estimate relative contribution of any individual taxa. Furthermore, we focused exclusively on the bacterial content of drinking water. We acknowledge that drinking water can contain protozoa, fungi and viruses, but we could not assess their potential role.

It is increasingly clear that microbial communities in the gastrointestinal tract, skin and airways are important contributors to health and disease.²⁰ However, we could not ascertain the host microbiome and its relationship to environmental exposures or outcomes which we measured. Given all these limitations, our findings should be considered as a proof-of-concept, rather than conclusive.

4.1.2 | Interpretation

Our findings that early-life exposure to high bacterial content in drinking water is associated with a reduced risk of asthma and other atopic phenotypes lend further support to the hygiene hypothesis^{21,22} and add to the growing number of potentially

TABLE 2 Distribution of lifetime and current symptoms by water supply type (public/individual) and location (urban/rural) among 541 children in the Phase 2 of the study

	Rural/Individual		Rural/Public		Urban/Individual		Urban/Public		Total		P-value
	n	%	n	%	n	%	n	%	n	%	
Lifetime											
Asthma	2/170	1.2	5/129	3.9	1/20	5.0	18/220	8.2	26/539	4.8	.014
Wheeze	31/170	18.2	26/130	20.0	9/21	42.9	73/218	33.5	139/539	25.8	.001
AD	11/170	6.5	14/130	10.8	2/19	10.5	43/219	19.6	70/538	13.0	.001
Rhinitis	19/168	11.3	54/130	41.5	6/19	31.6	79/218	36.2	158/535	29.5	<.001
Current											
Wheeze	7/170	4.1	15/130	11.5	7/20	35.0	52/219	23.7	81/539	15.0	<.001
AD	12/170	7.1	18/130	13.9	1/19	5.3	34/217	15.7	65/536	12.1	.049
Rhinitis	19/168	11.3	50/130	38.5	5/19	26.3	66/219	30.1	140/536	26.1	<.001
Sensitization	73/167	43.7	40/130	30.8	16/21	76.2	129/214	60.3	258/532	48.5	<.001

TABLE 3 Multivariate logistic regression analyses for the association between lifetime or current symptoms and the interaction of location with water supply type

	Asthma		Wheeze		Atopic dermatitis		Rhinitis	
	OR [95% CI]	P-value	OR [95% CI]	P-value	OR [95% CI]	P-value	OR [95% CI]	P-value
Lifetime								
Rural/Individual	0.14 [0.03,0.67]	.013	0.42 [0.25,0.72]	.001	0.2 [0.09,0.43]	<.001	0.17 [0.10,0.32]	<.001
Rural/Public	0.48 [0.16,1.49]	.206	0.46 [0.26,0.81]	.008	0.38 [0.19,0.78]	.008	0.95 [0.57,1.58]	.841
Urban/Individual	0.73 [0.09,6.06]	.772	1.42 [0.55,3.65]	.471	0.53 [0.12,2.41]	.411	1.00 [0.36,2.79]	.996
N	529		529		528		525	
	Wheeze		Atopic dermatitis		Rhinitis		Allergic sensitization	
	OR [95% CI]	P-value	OR [95% CI]	P-value	OR [95% CI]	P-value	OR [95% CI]	P-value
Current								
Rural/Individual	0.13 [0.06,0.31]	<.001	0.34 [0.16,0.70]	.004	0.21 [0.11,0.38]	<.001	0.63 [0.40,0.99]	.047
Rural/Public	0.35 [0.18,0.71]	.003	0.65 [0.33,1.29]	.219	1.00 [0.60,1.69]	.986	0.40 [0.24,0.66]	<.001
Urban/Individual	1.69 [0.60,4.72]	.319	0.33 [0.04,2.60]	.295	1.05 [0.35,3.11]	.934	1.83 [0.64,5.27]	.261
N	529		526		526		523	

Note: Reference group: children living in an urban location and receiving drinking water through public supply (Urban/Public). Age, sex and socio-economic status are included as covariates.

important exposures which may explain the lower prevalence of allergic diseases in rural environments.^{16,17,23,24} It is becoming increasingly evident that biodiversity of microbial exposure provides resilience against asthma and allergies.²⁵ High levels of sanitation, water treatment and food processing may lead to lower microbial exposure and reduced microbial diversity, thereby contributing to the increase in asthma and allergic diseases. Consistent with this, experimental studies have shown that germ-free mice, and mice with low-diversity microbiota, develop elevated serum IgE levels in early life.²⁶ In microbe-rich environments, exposure to microorganisms from different sources can occur through direct or indirect contact—via skin, respiratory tract or gut—and this environmental microbiome shapes the host microbiome and impacts upon disease development.^{27,28} However, it remains unclear which route of

exposure is important for specific diseases.²⁹ For example, it has been shown that nasal, but not throat microbiome, is associated with reduced risk of asthma.²⁷ Our data indirectly support the notion of the important role of the gut microbiome. The overall evidence on gut microbiota suggests that a more rapid maturation is associated with decreased asthma risk and that specific genera may be associated with protection (including *Veillonella*, *Lachnospira*, *Rothia* and *Bifidobacterium*),³⁰ whilst others (such as *Moraxella*²⁷ or *Neisseria*³¹) increase the risk. Our findings in relation to eczema are consistent with studies which have demonstrated the importance of fecal microbiota in eczema³² and the findings that relative abundance of immunomodulatory gut bacteria in the first year of life is associated with subsequent development of IgE-associated eczema.³³

TABLE 4 Multivariate logistic regression analyses for the association between bacterial load in drinking water (individual quantitative index of exposure quantified as the natural log of bacterial content in the drinking water in each child's first year of life) and lifetime/current symptoms

	Asthma		Wheeze		Atopic dermatitis		Rhinitis	
	OR [95% CI]	P-value	OR [95% CI]	P-value	OR [95% CI]	P-value	OR [95% CI]	P-value
Lifetime								
Cumulative bacterial load (ln)	0.77 [0.62,0.95]	0.016	0.92 [0.86,0.98]	0.013	0.85 [0.77,0.94]	0.001	0.78 [0.72,0.84]	<0.001
N	570		570		569		570	
	Wheeze		Atopic dermatitis		Rhinitis		Allergic sensitization	
	OR [95% CI]	P-value	OR [95% CI]	P-value	OR [95% CI]	P-value	OR [95% CI]	P-value
Current								
Cumulative bacterial load (ln)	0.79 [0.70,0.88]	<.001	0.90 [0.83,0.99]	.025	0.8 [0.74,0.86]	<.001	1.01 [0.96,1.07]	.645
N	569		567		566		563	

Note: Age, sex and socio-economic status are included as covariates.

Our data suggest that exposure in early life may be important and are consistent with results of a recent study in Finland which has suggested that exposure to sewage water during the first year of life, but not later, decreased the risk of IgE sensitization, emphasizing the importance of age as a modulator.³⁴ Infancy and early childhood seem to be crucial for the colonization of the gut, reaching an adult and relatively stable state at about 3-5 years of age,³⁵ paralleling host immune development.³⁶ This suggests an early-life "window of opportunity" when colonization has a potentially critical impact on health and disease.^{37,38} The temporal changes in microbiota may be important, and recent study has shown that there may be a time window before age 1 year in which colonization of the oropharynx with *Neisseria* is positively, and with *Granulicatella* species negatively, associated with subsequent wheezing.³¹

Our results are consistent with previous findings of the two studies from the Ethiopia and Latin America, which have reported that occurrence of atopy or atopic eczema is lower in subjects consuming river water compared to those consuming treated water from mains supply,^{39,40} and the previously highlighted study which compared Finnish and Russian Karelia.¹⁴ Our study population was more homogenous in relation to other putative risk factors compared to previous studies. It is also important to note that water is not boiled or filtered prior to drinking in our study area. Taken together, the results of these studies point out at the potential importance of the microbial content of drinking water as one of the important exposures which modulates the development of the immune system and impacts on the risk of immune-mediated diseases.

In conclusion, our results suggest that high microbial content in drinking water may be associated with a reduced risk of allergic diseases in childhood. However, these findings should be considered as hypothesis-generating and may facilitate the design of future research which should include mechanistic studies in human and animal models to gain insight into mechanisms of protection.^{41,42}

DATA AVAILABILITY STATEMENT

Raw data were generated at the Children's Hospital Srebrnjak, Zagreb, Croatia and County Hospital Požega, Croatia. Derived data supporting the findings of this study are available from the corresponding author [AC] on request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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ASSOCIATION OF BACTERIAL LOAD IN DRINKING WATER AND ALLERGIC DISEASES IN CHILDHOOD

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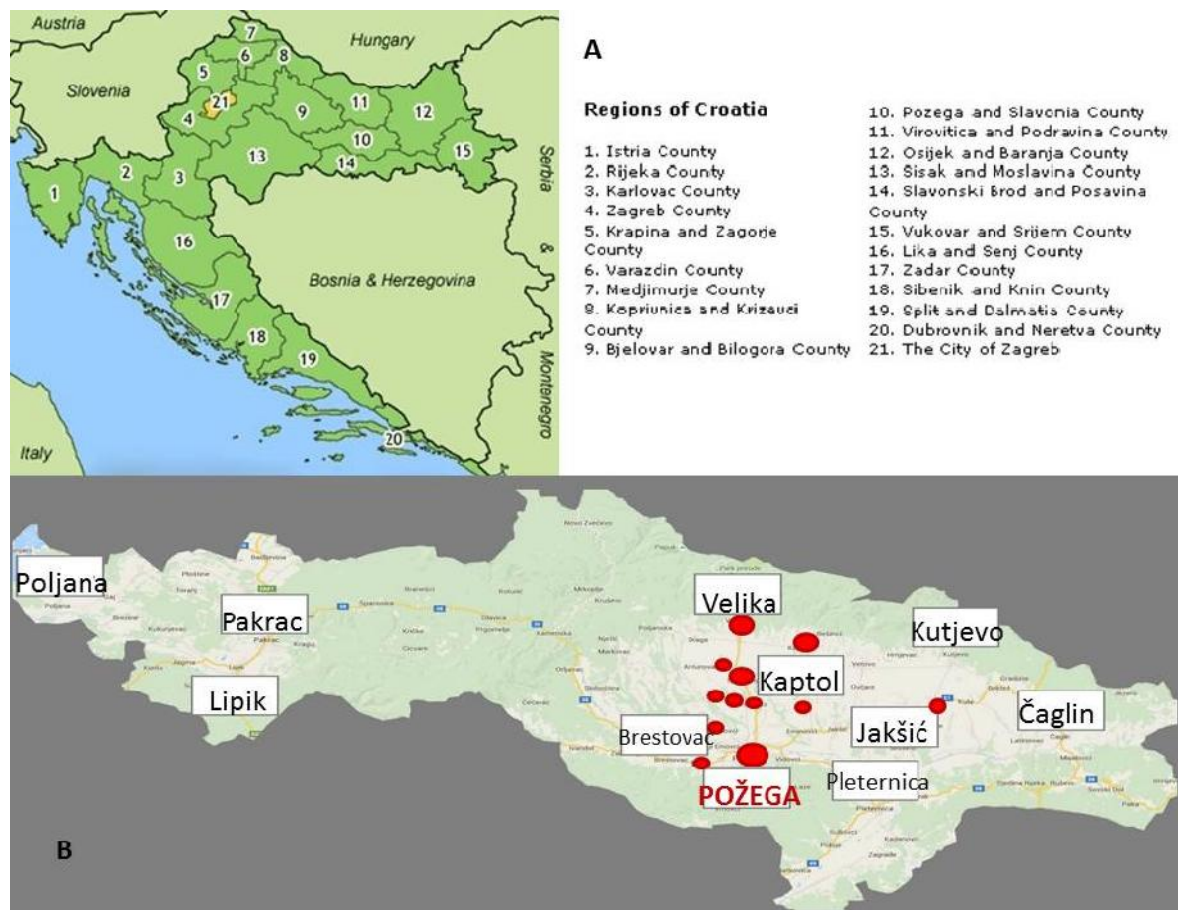
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ONLINE SUPPLEMENT

Figure S1. (A) Map of the Republic of Croatia with a list of its counties indicating the geographical position of the Požega-Slavonia county (10). (B) Map of the Požega-Slavonija county with some of its towns - Požega being the largest and the administrative center (described in the text) and locations of the participant's places of residence (red circles) (B).



METHODS

Data sources

The questionnaire included questions on symptoms and a diagnosis of allergic rhinitis (hay fever), respiratory symptoms, a previous diagnosis of asthma, symptoms and diagnosis of atopic eczema, family history of allergic diseases, children's age, sex, ethnic group, other demographic and lifestyle factors including number of siblings, passive smoking, questions about traffic and air pollution, housing style (e.g. crowdedness), indoor humidity, heating, airing, covering of floors, cleaning of carpets and floors, animal contact (livestock, poultry and pets), current medication, toilet facilities, drinking water supply, breast feeding and dietary factors.

Skin testing: The size of the wheal was documented as the mean of the longest diameter and the diameter perpendicular to it at its mid-point.

Definition of outcomes

Socio-economic status: The level of education was defined as the highest educational level attained, divided into four categories: (i) primary school or lower vocational or lower secondary education (as low); (ii) intermediate vocational education or intermediate/high school; (iii) higher vocational education or bachelor's degree and (iv) high vocational education or university degree. Family (parental) income was defined according to the average salary (the average monthly paid off net earnings per person in the Republic of Croatia; 730 Euro/month), and also divided into four categories: (i) less than average salary; (ii) round average; (iii) double higher than average salary and (iv) higher than double or net amount of salary higher than 1.500 Euro/month⁵⁴.

Analysis of the microbial content of drinking water

Data on water quality was collected in a 10 year period (1997-2007). These were courtesy of the Institute of Public Health Požega, Požega-Slavonia County. Water analysis involved all parameters of physicochemical adequacy (temperature, dullness, colour, smell, pH, ammonia, nitrite, nitrate, metal content - type A analysis) and microbiological quality of drinking-water (type B analysis). All analyses relied on the comparison of the water quality data with guideline values- maximum concentration allowed (Table S1).

Table S1. Indicators (parameters) for monitoring of physicochemical (type A analysis) and microbiological (type B analysis) quality for drinking water.

Type A analysis			Type B analysis		
Water quality parameter	Unit	Maximum concentration allowed (MCA)	Water quality parameter (indicator)	Unit	Maximum concentration allowed (MCA)
Alluminum*	mg/l	0.2	<i>E. coli</i>	Number/100 ml	0
Ammonia	mg/l	0.5	Total coliforms	Number/100 ml	0
Colour	mg/PtCo scale	20	<i>Enterococcus</i>	Number/100 ml	0
Conductivity	µS/cm /20° C	2500	Number of colonies 37° C	Number/1 ml	20
pH	pH units	6.5-9.5	Number of colonies 22° C	Number/1 ml	100
Smell		none	<i>C. perfringens</i> (spores included)***	Number/100 ml	0
Dullness	°NTU	4			
Nitrite**	mg/l	0.5			
Nitrate	mg/l	50			
Flavour		none			
Iron**	mg/l	200			
Chloride	mg/l	250			
Temperature	° C	25			
Disinfectant residues (eg. chlorite)	mg/l	400			

*Only when Al or Fe is used as a flocculating agent or when naturally present in the water. **Especially when chloramine is used as a disinfectant. ***Only if the water source is surface water or flows into one.

Minimal sampling frequency of water intended for human use from water supply systems, tanks or food processing and transport facilities, depending on the population size in the distribution net, is shown in Table S2. Relevant membrane filtration methods were used for the determination of viable counts of *Escherichia coli*, coliform bacteria, intestinal enterococci, *Salmonella*, *Pseudomonas aeruginosa* and *Clostridium perfringens* (given in Table S3).

Table S2. The frequency of sampling and monitoring.

Number of consumers within a supply zone	Amount of water delivered within a supply zone in m ³ /day	Type of analysis		Number of samplings	Number of posts on the distribution net	Annual sample number**
		"type A" analysis	"type B" analysis			
<500	<100	6	0	0.5 *	2	12
501 – 5.000	>100 – 1,000	10	2	1	4	48
5.001– 20.000	>1,000-4,000	20	4	2	5	120
20.001-50.000	>4,000-10,000	30	6	3	6	216

*in public water supply systems with less than 500 end users the water is sampled once every two months;

**annual sample number is calculated by summing the number of types of analysis and multiplying it by the number of posts on the distribution net.^{58,59}

Table S3. Microbiological indicators for drinking water

No.	Indicator	Method
1.	Total coliforms	ISO 9308-1:2000, membrane filtration*
2.	Total coliforms	HRN ISO 9308-2:1999, MPN**
3.	<i>Escherichia coli</i>	ISO 9308-1:2000, membrane filtration*
4.	<i>Escherichia coli</i>	HRN ISO 9308-2:1999, MPN**
5.	Enterococcus	COLILERT Enterococcus HRN EN ISO 7899-2:2000, membrane filtration*
6.	Number of colonies 37°C	HRN ISO 6222:2000*
7.	Number of colonies 22°C	HRN ISO 6222:2000*
8.	<i>Clostridium perfringens</i> (spores included)	Council Direct. 98/83/EC, Drinking Water Safety Regulation, NN 47/08 membrane filtration*
9.	<i>Pseudomonas aeruginosa</i>	HRN EN 12780:2005, membrane filtration**
10.	<i>Salmonella spp.</i>	HRN ISO 6340:2000, membrane filtration*

*Method is given in Drinking Water Safety Regulation and is used for analyses whose results are validated by the Regulation; **Method is not given in Drinking Water Safety Regulation NN 47/08, but is used for analyses whose results are not validated by the Regulation, Clause 15. Drinking Water Safety Regulation.

Table S4. Multivariate logistic regression analyses for the association between bacterial load in drinking water (individual quantitative index of exposure quantified as the natural log of bacterial content in the drinking water in each child's first and fifth year of life) and lifetime symptoms among 188 children with individual water supply who had measurable bacterial load. Age, sex, and socio-economic status are included as covariates.

	Wheeze		Atopic dermatitis		Rhinitis	
<i>Cumulative bacterial load (ln)</i>	<i>OR [95% CI], p value</i>		<i>OR [95% CI], p value</i>		<i>OR [95% CI], p value</i>	
<i>1st year of life</i>	0.61	0.021	0.85	0.611	0.85	0.521
	[0.40,0.93]		[0.45,1.59]		[0.52,1.40]	
<i>5th year of life</i>	0.99	0.960	1.91	0.103	0.99	0.981
	[0.64,1.53]		[0.88,4.16]		[0.59,1.68]	
<i>N</i>	188		188		186	