Histamine production in *Lactobacillus vaginalis* improves cell survival at low pH by counteracting the acidification of the cytosol

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Abstract

Histamine, one of the most toxic and commonly encountered biogenic amines (BA) in food, is produced by the microbial decarboxylation of histidine. It may accumulate at high concentrations in fish and fermented food. Cheese has some of the highest histamine concentrations, the result of the histidine-decarboxylase activity of certain lactic acid bacteria (LAB). The present work describes the nucleotide sequence and transcriptional organization of the gene cluster responsible for histamine biosynthesis (the *HDC* cluster) in *Lactobacillus vaginalis* IPLA 11064 isolated from cheese. The influence of histidine availability and pH on histamine production and the expression of the *HDC* cluster genes is also examined. The results suggest that the production of histamine under acidic conditions improves cell survival by maintaining the cytosol at an appropriate pH.

Keywords

Biogenic amines, histamine, *Lactobacillus vaginalis*, *HDC* cluster, gene expression, internal pH

1. Introduction

Histamine is a biogenic amine (BA) that can accumulate in food through the undesirable microbial decarboxylation of histidine. Histamine is commonly found at high concentrations in fish and fermented foods (EFSA, 2011; Shalaby, 1996). The consumption of food rich in histamine may trigger neurological, gastrointestinal and respiratory disorders (Alvarez and Moreno-Arribas, 2014; Ladero et al., 2010); indeed, histamine has been confirmed cytotoxic at food concentrations (Linares et al., 2016). A synergistic effect with tyramine, another BA frequently detected in cheeses and other fermented foods, is known to occur (del Rio et al., 2017). Histamine is the only BA for which maximum legal limits have been established - although only for fish and fish products (200-400 mg/kg, established by European Union Commission Directives 2073/2005 and 1019/2013, and 50 mg/kg, established by the US Food and Drug Administration (FDA, 2001)). It has been detected in cheese, however, at concentrations far exceeding these suggested limits (Fernandez et al., 2007).

In fermented foods, histamine is mainly produced by certain LAB (Linares et al., 2011; Shalaby, 1996), which catalyze the decarboxylation of histidine via the action of the enzyme histidine decarboxylase (HdcA). The secretion of histamine from the cytosol in exchange for histidine is undertaken by the antiporter HdcP. These proteins are encoded by the genes *hdcA* and *hdcP* respectively, both of which are found in the *HDC* cluster. The *HDC* cluster can also include the gene *hdcB*, which codes for a protein that catalyses the maturation of HdcA (Trip et al., 2011), and *hisS*, which encodes a putative histidyl-tRNA synthetase (Martin et al., 2005). The *HDC* cluster has been described in several species of LAB, including *Lactobacillus saerimneri*

(Romano et al., 2013), *Lactobacillus parabuchneri* (formerly *L. buchneri*) (Diaz et al., 2016; Martin et al., 2005), *Lactobacillus hilgardii* (Lucas et al., 2005), *Lactobacillus vaginalis* (Diaz et al., 2015), *Tetragenococcus halophilus* (Satomi et al., 2008), and *Streptococcus thermophilus* (Calles-Enriquez et al., 2010). It can be also found in the genome of *Lactobacillus reuteri* (Gene ID: 5189585) and *Tetragenococcus muriaticus* (Gene ID: 13919182).

Amino acid decarboxylations are proton-consuming reactions that may provide acid resistance by helping to maintain the intracellular pH (Kanjee and Houry, 2013). Certainly, tyrosine decarboxylation improves the survival of *Enterococcus faecalis* under acidic conditions (Perez et al., 2015), and when histidine is present the heterologous expression of the corresponding decarboxylase pathway in *S. thermophilus* and *Lactococcus lactis* increases their survival of acid stress (Trip et al., 2012). This suggests that pH might regulate amino acid decarboxylation via gene expression and/or enzymatic activity. An increase in histidine decarboxylation activity at acidic pH has been reported for *L. saerimneri* and *S. thermophilus* (Schelp et al., 2001; Tabanelli et al., 2012). Histamine accumulation is also influenced by environmental factors such as, temperature, salt concentration, thermization, and the presence of ethanol (Landete et al., 2008b; Linares et al., 2012a). Understanding the factors that influence histamine accumulation in foods such as cheese is key if it is to be reduced.

The present work examines the transcriptional organization of the *HDC* cluster in the cheese isolate *L. vaginalis* IPLA 11064, a representative histamine-producing strain. The influence of histidine availability and pH on histamine production was also examined, and the role played by histamine in the survival

of this strain under acidic conditions, i.e., those found in cheese matrices -
investigated.

2. Materials and Methods

2.1. Strains and growth conditions

L. vaginalis IPLA 11064, a histamine-producing strain isolated from cheese (Diaz et al., 2015), was grown in MRS (Oxoid, Basingstoke, UK) at 37°C without aeration.

To check the effect of histidine concentration on gene expression and histamine production, cells were grown in 50 mL of chemically defined medium (CDM) (Poolman and Konings, 1988) supplemented with different histidine concentrations (CDM+His), and incubated at 37°C for 14.5 h. To examine gene expression and histamine production under controlled pH conditions, cells were cultivated in a Six-Fors bioreactor (Infors AG, Bottmingen, Switzerland) in MRS supplemented with 10 mM histidine (MRS+His) for 10 h. Sterile fermentation vessels were aseptically filled with 400 mL MRS+10 mM His at pH 4.0, 5.0, 6.0 and 7.0. The pH was maintained during the culture period by the automatic addition of 2 N NaOH or 2 N HCl. The reactor was maintained at 37°C, with stirring at 50 rpm and zero air input.

2.2. RNA isolation

L. vaginalis cells grown under the above-described conditions were harvested by centrifugation. Total RNA was extracted using the TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA) as previously described (Linares et al., 2009). To eliminate any DNA, RNA samples were treated with Turbo™ DNase (ThermoFisher Scientific, Waltham, MA, USA) as described by the manufacturer. The total RNA concentration was determined using an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, USA).

2.3. Reverse transcription PCR (RT-PCR)

cDNA was synthesized from 0.5 µg of total RNA using the reverse transcription (RT) iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's recommendations. Three µL of the cDNA suspension were used as a template for the PCR reactions, adding 400 nM of each primer (Table 1), 200 µL of dNTP, 1 U of Taq polymerase (DreamTaq, Fermentas, Vilnius, Lithuania) and the reaction buffer. Amplifications were performed over 35 cycles (95°C for 30 s, 50°C for 45 s and 72°C for 1 min) and the resulting fragments electrophoretically separated in 1% agarose gels in TAE buffer (40 mM TRIS/acetate, 1 mM EDTA; pH 8.0). The absence of contaminating DNA in the DNase-treated RNA samples was confirmed by PCR performed under the same conditions but using samples of RNA from before the reverse transcription step as a template.

2.4. Quantification of gene expression by RT-qPCR

Gene expression was examined by reverse transcription quantitative real-time PCR (RT-qPCR) using a 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) and employing the SYBR® Green PCR Master Mix (Applied Biosystems). Amplifications were performed with specific primers (Table 1), the design of which was based on the genome sequence of *L. vaginalis* ATCC 49540 (GenBank accession no. ACGV01000000) (performed using Primer Express software [Applied Biosystems]). Fourfold dilutions of the cDNA samples were used as a template (4 µL) in a final volume of 20 µL. *RecA* and *gap* genes were used as internal controls to normalize the RNA concentration. The linearity and amplification efficiency of the reactions were tested for each primer pair using five 10-fold serial dilutions of genomic *L. vaginalis* IPLA 11064 DNA. The primer concentration used for RT-qPCR analysis was 200 nM for LvagRecAF/LvagRecAR, 300 mM for LvagGapF/LvagGapR, and 800 nM for hdcQLvF/hdcQLvR, qhisSLvF/qhisSLvR and qhdcPLvF/qhdcPLvR. No-template samples were included in each run as negative controls. The cycling settings were the defaults set by Applied Biosystems. Threshold cycle (Ct) values were calculated automatically using 7500 Software v.2.0.4 (Applied Biosystems). The abundance of messenger RNA (mRNA) species was calculated following the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). The condition with the lowest level of expression in each experiment was selected as the calibrator. In all cases, RT-qPCR analysis was performed on RNA purified from three independently grown cultures.

2.5. Quantification of histamine

Histamine in the supernatants obtained by centrifugation (8000 x g for 10 min) of the cultures grown under the different experimental conditions was quantified by ultra-high performance liquid chromatography (UHPLC). All supernatants were filtered through 0.45 µm polytetrafluoroethylene (PTFE) filters (VWR, Barcelona, Spain), derivatized with diethyl ethoxymethylenemalonate (Sigma-Aldrich), and the histamine content quantified using the UPLC™ system (Waters, Milford, MA, USA) as previously described (Redruello et al., 2013). Data were acquired and analyzed using Empower 2 software (Waters). The histamine concentrations provided are the mean of three independent experiments.

2.6. Determination of cell viability under acid shock

Following Romano et al. (2014) with some modifications, cells were grown on MRS until early-exponential phase (OD 0.7 approximately) and harvested by centrifugation (3000 g x 10 min). They were then washed in sterile PBS at pH 5.0 and resuspended at OD 0.2 in PBS with or without histidine (10 mM final concentration) at pH 4.0, 3.0 or 2.0. These cell suspension were then incubated at 37°C for 4 h. The aliquots were serially diluted and 100 µL of each dilution plated on MRS and incubated at 37°C under anaerobic conditions (10% H₂, 10% CO₂ and 80% N₂) in a Mac 500 anaerobic workstation (Don Whitley Scientific, Shipley, UK) for 48 h. The initial viable cell concentration (in CFU/mL) was calculated from cell suspensions in PBS at pH 5 without histidine before incubation. At the end of the experiment, the amount of histamine was

quantified by UHPLC as described in 2.5. All experiments were performed in triplicate.

2.7. Measurement of internal pH

Internal pH measurements were taken using the internally conjugated fluorescence pH probe carboxyfluorescein succinimidyl ester (cFSE, Sigma-Aldrich) as previously described (Perez et al., 2015). Briefly, cells were grown in MRS until mid-exponential phase, harvested by centrifugation and washed twice in CPK buffer (sodium citrate 50 mM, disodium phosphate 50 mM, potassium chloride 50 mM) at pH 7.0. They were resuspended in CPK buffer at the pH required (ranging from 4.5 to 7.5) and incubated at 37°C for 30 min in the presence of the precursor probe carboxyfluorescein diacetate succinimidyl ester. The cells were then washed and resuspended in CPK buffer with 10 mM glucose at the corresponding pH and incubated at 37°C for 15 min. The cells were then washed again in CPK buffer at the required pH. Finally, the treated cells were resuspended in 100 μ L of CPK buffer supplemented with 10 mM histidine (final concentration) at the required pH, and under the same conditions but without histidine (controls). Fluorescence intensities were measured over 20 min (at intervals of 0.45 s) using a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, CA, USA) using previously described excitation and emission values (Breeuwer et al., 1996). Background fluorescence levels were determined by examining the non-fluorescent control cells. Internal pH values were calculated from the ratio of the fluorescent signal obtained at 490/440 nm, using a calibration curve constructed employing

210 buffers at pH 4.5-7.5, and after equilibrating the internal (pH_{in}) and external
211 (pH_{out}) pH with valinomycin (1 μM) and nigericin (1 μM). The value given for
212 each condition is the average of three independent replicates (each the mean of
213 values obtained over 15 min of monitoring).

215 2.8. Statistical analysis

216 Means were compared using the Student *t* test. Significance was set at $p < 0.05$.

218 3. Results

219 3.1. Effect of histidine concentration on histamine production

220 *L. vaginalis* was grown at 37°C for 14.5 h in CDM supplemented with different
221 histidine concentrations (0, 0.05, 0.1, 0.5, 1, 5 and 10 mM). In the absence of
222 histidine, no histamine was detected. The production of histamine was
223 observed after the addition of 0.1 mM histidine, and increased as the
224 concentration of histidine increased, reaching a stable maximum at 5 mM
225 histidine (Figure 1).

227 3.2. Low pH increased histamine biosynthesis

228 The influence of acidic pH on histamine production was studied by measuring
229 growth (via optical density) and the production of histamine itself at different
230 pHs. Figure 2A shows that histamine production was greater at pH 4.0 and 5.0
231 and less intense at higher pHs (minimal production reached at pH 7.0). The

OD₆₀₀ at pH 4.0 (Figure 2B) was lower than that recorded for the other pH conditions assayed over the entire growth curve, indicating that the greater production of histamine at pH 4.0 was not due to greater growth. Increased gene expression at this pH, or greater histidine decarboxylase activity, are the likely alternatives.

3.3. Transcriptional organization of the *HDC* cluster

To study the expression of the *HDC* cluster genes, the transcriptional organization of the cluster was first determined. cDNA of the total RNA of cultures grown in CDM with histidine (10 mM) and without was RT-PCR amplified. Internal fragments of the four genes from the *HDC* cluster were amplified (data not shown) using five additional pairs of primers (Table 1) designed to amplify the intergenic and flanking regions of the *HDC* cluster (Figure 3A and S1). As expected, no RT-PCR amplification was seen for the intergenic regions between *hdcP* and the gene located upstream (a gene that codes for a C4-dicarboxylate anaerobic carrier not expected to be involved in histamine synthesis), nor between *hisS* and the gene located downstream (which codes for a major facilitator superfamily transporter not expected to be involved in histamine synthesis either) (Figure 3). Amplicons were only obtained for the intergenic region between *hdcA* and *hdcB* (Figure 3B), indicating that *hdcA* and *hdcB* are co-transcribed. The mRNA covering *hdcA* and *hdcB* starts with a putative *hdcA* promoter and ends at the putative rho-independent terminator hairpin downstream of *hdcB* ($\Delta G_{hdcB} = -13.5$ kcal/mol) (Figure 3A and S1). No amplicons were obtained for the *hdcP-hdcA* or *hdcB-hisS* intergenic regions, indicating that *hdcP* and *hisS* are individually transcribed as monocistronic mRNAs covering the region from their own putative promoters to

the putative rho-independent terminator hairpins ($\Delta G_{hdcP} = -10.3$ kcal/mol and $\Delta G_{hisS} = -22.8$ kcal/mol) located downstream of them (Figure 3A and S1). The same results were obtained when cells were grown in CDM without histidine (data not shown) indicating the existence of a basal transcription level for the genes of the *HDC* cluster.

3.4. Effect of histidine concentration and pH on the expression of genes in the *HDC* cluster

To examine the effect of the histidine concentration on *HDC* cluster gene expression, the strain was cultured in CDM with histidine concentrations ranging from 0 to 10 mM. *hisS*, *hdcA* and *hdcP* gene expression was then quantified by RT-qPCR after 14.5 h of incubation (a point at which the cells were in exponential growth and had not exhausted the histidine in the medium). Figure 4 shows that maximum *hisS* expression occurred in the absence of, or low concentrations of, histidine. The expression of *hisS* diminished when the histidine concentration was 1 mM or higher. In contrast, the expression of *hdcA* correlated positively with the concentration of histidine. Induction was enhanced up to a histidine concentration of 0.5 mM, after which no significant increase was observed (Figure 4). The same cDNAs used in previous experiments were used to quantify *hdcP* expression, which increased with the concentration of histidine, reaching a maximum at 0.5 mM histidine, after which the expression was stable (Figure 4).

The expression of *hdcA* was quantified by RT-qPCR using cultures grown in CDM medium supplemented with 10 mM histidine, at pH 4.0, 5.0, 6.0 or 7.0. No significant differences in expression were recorded (data not shown).

These results show that histidine concentration, but not acidic pH, controls the transcription of the genes in the *HDC* cluster.

3.5. Histamine biosynthesis improves cell survival under acidic conditions

The effect of histidine decarboxylation on *L. vaginalis* survival at pH 2.0, 3.0, and pH 4.0 was using examined cells growing in MRS. The presence or absence of histidine had no effect on survival at pH 4 (Figure 5). However, the presence of 10 mM histidine increased survival at pH 3 from 71.19 % to 100%. The largest effect was observed at pH 2: no cells survived in the absence of histidine, but more than 70% of the population survived in the presence of 10 mM histidine (Figure 5). In the samples containing histidine, the production of histamine was quantified at the end of the experiment and was found to be greater at pH 3.0 (0.763 mM) and pH 2.0 (0.627 mM) than at pH 4.0 (0.166 mM) (Figure 5).

3.6. Histamine biosynthesis counteracts the acidification of the cytosol under acidic conditions

To determine whether the effect of histamine synthesis on survival under acid stress conditions is related to the ability to maintain the intracellular pH, the cytosolic pH was monitored at pH 4.5-7.5 in the presence/absence of histidine

(10 mM). Figure 6 shows that in the presence of histidine the variation in the intracellular pH at different extracellular pHs was less than in its absence. Even at the lowest assayed external pH (4.5), the internal pH was above 6.5 in the presence of histidine. These results suggest a role for histamine in pH homeostasis, counteracting the intracellular acidification caused by reductions in the external pH.

4. Discussion

Histamine can accumulate at high concentrations in cheese (Fernandez et al., 2007), and the ingestion of such food can pose a health risk to consumers. Although, there is no legal limit for the presence of histamine in fermented dairy products, it is recommended that its accumulation be avoided (EFSA, 2011; Ladero et al., 2010). Determining the factors that influence the accumulation of histamine, including the presence of histamine-producing microorganisms, is essential if we are to apply appropriate food safety measures. Certainly, knowledge of the physiological role and molecular regulation mechanisms of histamine in microorganisms isolated from cheese is essential. The present work helps in this respect by describing the effect of substrate availability and pH on histamine and *HDC* cluster gene expression, and the acid-resistance role of histamine in *L. vaginalis* strain IPLA 11064 (Diaz et al., 2015).

Different transcriptional organizations of the *HDC* cluster have been reported for different LAB species. In the present *L. vaginalis* IPLA 11064 strain, a bicistronic mRNA covering *hdcA* and *hdcB* was seen, while *hdcP* and *hisS* were transcribed as single monocistronic mRNAs, both in the presence and absence

of histidine. In *S. thermophilus* CHCC1524, however, *hdcA* is transcribed as a monocistronic mRNA, or as part of a polycistronic mRNA that includes the *hdcA*, *hdcP* and *hdcB* genes (Calles-Enriquez et al., 2010). In *L. parabuchneri* (formerly *L. buchneri*) B301, *hdcA*, *hdcB* and *hisS* are cotranscribed in a polycistronic mRNA in the absence of histidine, while *hdcP* is transcribed independently. In the presence of histidine, however, the polycistronic mRNA only includes *hdcA* and *hdcB*, while *hdcP* and *hisS* are transcribed independently as monocistronic mRNAs (Martin et al., 2005). The transcriptional organization of *L. vaginalis* IPLA 11064 is therefore similar to that of *L. parabuchneri* B301 in the presence of histidine. The regulation of *hisS* by histidine in this latter strain is due to the presence of a leader region upstream of the start codon of the gene, which has the sequence features of a tRNA-mediated antitermination system (Martin et al., 2005). The sequence features that determine the termination-antitermination system for the regulation of *hisS* were not found *in silico* in *L. vaginalis* IPLA 11064 (supplementary Figure S1). The lack of these features might explain the observed difference with *L. parabuchneri* B301 in the absence of histidine. Although *L. vaginalis* IPLA 11064 lacked this regulation system, the expression of *hisS*, which encodes a tyrosyl-tRNA synthetase-like enzyme, was somehow repressed by high concentrations of histidine (Figure 4). As described for other aminoacyl-tRNA synthetase-like genes in other aminoacyl decarboxylation gene clusters (Linares et al., 2012b; Perez et al., 2015), the transcription of *hisS* was inversely correlated with the concentration of histidine (Figure 4).

The influence of histidine on the biosynthesis of histamine has been studied in several species (Lonvaud-Funel and Joyeux, 1994; Pessione et al., 2005). In

the present work, as more histidine became available the production of histamine increased (Figure 1). This result is of importance with respect to dairy fermentations: milk has a high protein content and proteolysis during the ripening of cheeses results in high concentrations of free histidine for conversion into histamine (Joosten, 1987; Linares et al., 2011, 2012a). The present analysis of *hdcA* expression showed that histidine enhances the expression of *hdcA* and *hdcP* in *L. vaginalis* IPLA 11064, which translates into an increase in histamine production (Figure 1 and Figure 4). This has also been reported for other LAB species such as *L. hilgardii* 464, *P. parvulus* P270 and *O. oeni* 4042 (Landete et al., 2006), and for *S. thermophilus* strains found in the dairy environment (Calles-Enriquez et al., 2010; Rossi et al., 2011). The expression of *hdcA* in *L. vaginalis* IPLA 11064 was induced by a histidine concentration as low as 0.05 mM, even though *L. vaginalis* IPLA 11064 was found to be auxotrophic for histidine (data not shown), indicating that the cells decarboxylated this histidine even though it was needed for protein biosynthesis.

pH has also been described to have an influence on the accumulation of histamine (Landete et al., 2008a). The present results show that an acidic pH enhances histamine biosynthesis in *L. vaginalis* IPLA 11064. Histamine production at pH 4 and 5 was significantly greater than at pH 6 and 7 (Figure 2A). Moreover, the growth recorded at pH 4 was less than that recorded under the other conditions assayed. This indicates that the greater production of histamine at pH 4 is not due to increased growth but to an increased histamine production rate. These results agree with the greater production of histamine at acidic pHs recorded for other histamine-producing bacteria such as *O. oeni*

(Lonvaud-Funel and Joyeux, 1994), *L. hilgardii* and *P. parvulus* (Landete et al., 2006). The increase in histamine production at acidic pH might be due to the induction of gene expression or more intense enzymatic activity. In the present *L. vaginalis* IPLA 11064 strain, pH had no effect on *hdcA* expression, indicating that the increased production of histamine at low pH is explained by the activation of HdcA, as described for *L. saerimneri* 30a (Schelp et al., 2001) and *L. hilgardii* (Landete et al., 2008b). Acidic pH has also been shown to have no influence on the transcription of *hdcA* in *S. thermophilus* (Calles-Enriquez et al., 2010). This contrasts with that observed for other decarboxylation clusters involved in the formation of tyramine in *E. faecalis* and *E. durans*, in which the tyrosine decarboxylase gene (*tdcA*) is transcriptionally activated at acidic pH (Linares et al., 2009; Perez et al., 2015).

The decarboxylation of amino acids has been described to help bacteria resist acidic environments (Fernandez de Palencia et al., 2011; Kanjee and Houry, 2013; Pereira et al., 2009) - such as those found in fermented products - via the maintenance of the internal pH (Perez et al., 2015; Romano et al., 2014; Trip et al., 2012). In the present work, in which *L. vaginalis* IPLA 11064 cells were incubated under acidic conditions, the presence of histidine increased the survival rate (Figure 5). Indeed, histamine production was greater at pH 3.0 and 2.0 than at pH 4.0 (Figure 1). The results also suggest that the protection afforded by histamine was achieved via the maintenance of the cytosol pH (Figure 2). These results agree with those obtained by other authors who also indicate histidine decarboxylation to help maintain the internal pH (Perez et al., 2015; Romano et al., 2014; Trip et al., 2012).

In conclusion, the decarboxylation of histidine provides a means by which *L. vaginalis* IPLA 11064 can resist acidic external pHs, i.e., by counteracting the acidification of the cytosol. The production of histamine from histidine is influenced by the histidine concentration, but it is enhanced at such pHs. Therefore, the *HDC* pathway could be considered an acid stress resistance mechanism for *L. vaginalis* that provides important advantages when facing acidic environments, such as those encountered in dairy fermentations. However, it should be noted that the product is histamine, a toxic compound that has food safety implications.

5. Declaration of competing interest

The authors declare no conflict of interest.

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8. Figure legends

Figure 1. Production of histamine by *L. vaginalis* IPLA 11064 in CDM medium supplemented with 0, 0.05, 0.1, 0.5, 1, 5, or 10 mM histidine for 14.5 h. Supernatants were analyzed by UHPLC to determine the histamine concentration of the extracellular medium.

Figure 2. A) Influence of pH (4.0, black bar; 5.0, white bar; 6.0, grey bar and 7.0, dotted bar) on histamine production in *L. vaginalis* IPLA 11064. Asterisks indicate a significant difference in histamine production ($p < 0.05$) compared with pH 4.0 (Student *t* test). B) Growth curves of *L. vaginalis* IPLA 11064 at pH 4.0 (rhombus), 5.0 (square), 6.0 (triangle) and 7.0 (circle).

Figure 3. Genetic organization and transcriptional analysis of the *HDC* cluster of *L. vaginalis* IPLA 11064. A) Organization of the *HDC* cluster of *L. vaginalis*. Possible promoters are indicated by curved arrows and termination regions are represented by hairpins. Intergenic regions amplified by RT-PCR are represented by the letters A-E and the expected mRNAs are indicated. B) RT-PCR amplification of the intergenic regions: fragment A (*C4-hdcP*), fragment B (*hdcP-hdcA*), fragment C (*hdcA-hdcB*), fragment D, (*hdcB-hisS*) and fragment E (*hisS-MFS*). Negative control (-) consisting of DNase-treated RNA samples without reverse transcriptase, and positive controls (+) with chromosomal DNA. *M* molecular weight markers (GeneRuler DNA ladder mix, Fermentas).

Figure 4. Quantification of gene expression by RT-qPCR. Effect of different histidine concentrations on the expression of *hdcA* (grey bars), *hdcP* (black bars) and *hisS* (white bars), in *L. vaginalis* IPLA 11064 grown in CDM supplemented with histidine. The lowest expression level for each gene was normalized to 1 and used as the reference condition. Bars with the same letter indicate significant differences with respect to the no-histidine condition (Student *t* test).

Figure 5. Cell survival and histamine production in *L. vaginalis* IPLA 11064 exposed to acid shock in the presence and absence of histidine. Survival (%) of *L. vaginalis* IPLA 11064 incubated for 4 h in the presence (black bars) and absence (grey bars) of 10 mM histidine. Survival was measured by plate counts. Values are expressed as a percentage of the control value (the 100% control value for untreated cells corresponded to 1.49×10^7 CFU/mL). Asterisks indicate a significant difference ($p < 0.05$; Student *t* test). The production of histamine in the presence of histidine under each condition is indicated. The results are the mean $\pm$ SD of experiments performed in triplicate.

Figure 6. Variation of the intracellular pH (pH_{in}) at different extracellular pHs (pH_{out}) measured using a cFSE probe in resting *L. vaginalis* IPLA 11064 cells in the presence (black circles, 10 mM histidine) and absence (white circles, control condition) of histidine. Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.005$; Student *t* test).

9. Tables

Table 1. Primers used in this study

Primer	Nucleotide sequence (5'-3')	Function	Reference
hisLvC4F	TTTCATTGTGCCATCTTCTTCCG	<i>C4-hdcP</i> RT-PCR	(Diaz et al., 2015)
hisPLv5R	ATCACTATCGGCCATGTTGC	<i>C4-hdcP</i> RT-PCR	This study
hisPLv6F	AAGCACCGTCAATGGGCTGG	<i>hdcP-hdcA</i> RT-PCR	This study
hdcQLvR	TTAGTTTCGGCACGATCGTATG	<i>hdcP-hdcA</i> RT-PCR and <i>hdcA</i> expression analysis	This study
hisLr2F	TGCTGAGTGTGGTCAAGATGC	<i>hdcA-hdcB</i> RT-PCR	(Diaz et al., 2015)
hisBLv1R	ACTGGTAATGAGTGAAGCAGG	<i>hdcA-hdcB</i> RT-PCR	This study
hisALv2F	CCAGTGAATTTAAACAAGCG	<i>hdcB-hisS</i> RT-PCR	This study
hisSLv2R	ATGGCTTAGCATATTCAGGTCC	<i>hdcB-hisS</i> RT-PCR	This study
hisSdsLvF	CTGAAGCGGACAGCAGTGAGAAAGG	<i>hisS-MFS</i> RT-PCR	(Diaz et al., 2015)
hisLv1	CCCTACATTAAATTACATGTCC	<i>hisS-MFS</i> RT-PCR	(Diaz et al., 2015)
hdcQLvF	TGGTGTTCTGTGACAAGACTGATG	<i>hdcA</i> expression analysis	This study
qhisSLvF	CGGTGGTTACACGACAATTTGT	<i>hisS</i> expression analysis	This study
qhisSLvR	CCACCAATTTGCGAAATCAAT	<i>hisS</i> expression analysis	This study
qhdcPLvF	TGATAAATCCCAAGCTGCAAAA	<i>hdcP</i> expression analysis	This study
qhdcPLvR	CATCGCACTAACACACAAAGCA	<i>hdcP</i> expression analysis	This study
LvagRecAF	TGATCAACGAAAAGCAGCACTT	<i>recA</i> expression analysis	This study

LvagRecAR	GATGGAGCCCTTACCGAAATT	<i>recA</i> expression analysis	This study
LvagGapF	TCGTTTTGATGGCGAAGTTTC	<i>gap</i> expression analysis	This study
LvagGapR	CAGGGTATTCCTTACCATCAACAA	<i>gap</i> expression analysis	This study

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Figure 1

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Figure S1

Figure S1. *L. vaginalis* IPLA 11064 sequences of the *hdcP* upstream region (A), *hdcP-hdcA* (B), *hdcB-hisS* (C) intergenic regions, and the *hisS* downstream region (D). The putative -10 and -35 promoter sequences, ribosome binding site sequences (RBS) and rho-independent terminator sequences (dashed lines) are indicated. The nucleotides of genes appear in gray backgrounds and start and stop codons in bold. The transcriptional direction is represented by dotted arrows.